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14. ABSTRACT With disease progression, the majority of the prostate cancers would eventually evolve into lethal aggressive hormone refractory disease; therefore, there are needs for developing new strategies to prevent the disease progression. We have identified a cross-talk between vitamin D and COX-2 inhibitor, two chemopreventative agents for prostate cancer, and conducted series investigations of their anti-prostate cancer effects with the funding support from DOD, which have led to generation of three publications. First, we identified the molecular mechanism by which vitamin D inhibits prostate cancer angiogenesis through IL-8, finding a strong correlation of IL-8 expression with prostate cancer disease progression, therefore, inhibition of IL-8 by vitamin D supports the chemotherapeutic effects of vitamin D in preventing prostate cancer progression. Second, we studied the vitamin D-based combination with docetaxel therapy. Docetaxel is the only treatment shown to improve overall survival in hormonal refractory prostate cancer patients (HRPC); however the survival benefit is modest. Treatment with docetaxel in combination with the active form of vitamin D has shown promising results in prostate specific antigen (PSA) response, time to progression and survival in HRPC patients. Our detailed mechanism of this combination therapy was studied to provide a further therapeutic design. Third, the mechanism(s) of elevation of COX-2 expression in late stage of disease and its contribution to the cancer progression were further explored. We found that androgen/AR signals suppressed COX-2 activity, therefore the blockage of androgen signals in complete androgen blockage therapy would then consequently result in elevated COX-2 expression and promote the disease progression. The clinical use of COX-2 inhibitors has recently become controversial due to cardiovascular complications associated with the use of COX-2 inhibitor for prolonged periods of time. Our studies supported that suppression of COX-2 would be beneficial to the prostate cancer therapy, suggesting an urgent a need to develop more potent COX-2 inhibitors with fewer side effects.

15. SUBJECT TERMS
Prostate Cancer Therapy, vitamin D and COX-2 inhibitor

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To Investigate the Therapeutic Effects of the COX-2 Inhibitor NS-398 as a Single Agent, and in Combination with Vitamin D, *In Vitro* and *In Vivo*

Introduction: The incidence of prostate cancer has increased rapidly and has become the most common malignancy of men in many Western nations. Most prostate cancer initially responds to androgen ablation treatment, however, eventually it relapses to an androgen-independent state. Therefore, much effort is needed towards understanding the mechanisms involved in development and progression of prostate cancer and developing new strategies for prevention and treatment. Results of recent epidemiologic and animal model studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs), which prevent biosynthesis of prostagladins through inhibition of COX activity, act as chemopreventative agents. In our preliminary studies we examined the expression of COX-2 in prostate cancer tissue arrays, which consist of normal, BPH, PIN, and low-and high-grade prostate tumor samples and found that COX-2 expression is significantly higher in tumorsr than in normal or BPH prostate samples and the tendency of COX-2 expression in prostate cancer cells is consistent with prostate cancer cell lines, implying COX-2 expression in prostate cancer might be up-regulated during cancer progression. Treatment of prostate cancer cells with a selective COX-2 inhibitor, NS-398, induces VDR expression, and thus might result in increasing the vitamin D sensitivity of such cells. In return, treatment of prostate cancer cells with vitamin D (1,25-VD) results in reduction of COX-2 mRNA expression, but not COX-1 expression. We hypothesize that combining vitamin D and a COX-2 inhibitor in the treatment of prostate cancer will be beneficial. We will examine the effects of combination of 1,25-VD, its analog (EB1089), and the COX-2 inhibitor NS-398, compared with single agent treatment, on prostate cancer growth, apoptosis, invasion, angiogenesis, and neuroendocrine differentiation *in vitro* and *in vivo*, through the following Aims. AIM 1: Evaluation of the molecular mechanism of COX-2 inhibitor NS-398 action on the growth of prostate cancer cells. AIM 2: Evaluation of the effects of 1,25-VD and its analogs in combination with COX-2 inhibitor on progression of prostate cancer cells. AIM 3: Evaluation of the underlying mechanism of the bi-directional regulatory pathways between the COX-2 inhibitor NS-398 and vitamin D. AIM 4: Evaluation of the effects of COX-2 inhibitor, administered in combination with vitamin D compounds, on prostate cancer progression and invasion in vivo. Our proposed schedule for completion of this proposal included the following tasks during the past 12 months. Task 1: Determine whether the inhibition of growth mediated by NS-398 is a COX-2-dependent pathway (Month 1-6). Measure the COX-2 protein level and prostaglandin secretion level. Restore NS-398 effect by adding prostaglandin. Task 2: Determine the NS-398 mediated anti-tumor pathways (anti-proliferation, anti-invasion, anti-tumorigenesis, and anti-angiogenesis (Month 1-12). Task 3: Determine the molecules that are responsible for NS-398 anti-tumor action (RT-PCR, real-time PCR analysis of expression of known genes involved in NS-398 action, and DNA array analysis) (Month 6-18).

Body:

Our progress and completion of Task 1-5 have led to generation of three publications.

1) The first paper was published by *Cancer Letters*, 247, 122-129, 2007 entitled “Docetaxel-induced growth inhibition and apoptosis in androgen independent prostate cancer cells are enhanced by 1α,25-dihydroxyvitamin D3”. Docetaxel (Taxotere) is the only treatment shown to improve overall survival in hormonal refractory prostate cancer (HRPC) patients; however the survival benefit is

modest with an improved median survival of only 2.5 months. Obviously, greater improvements in treatment are needed. Treatment with docetaxel in combination with the active form of vitamin D has shown promising results in prostate specific antigen (PSA) response, time to progression, and survival in HRPC patients. The detailed mechanism of this combination therapy was studied in this paper to provide a further therapeutic design.

The second paper was published by *Carcinogenesis,* 27 (9):1883-1893, 2006 entitled "1α. 25-dihydroxyvitamin D3 suppresses interleukin-8-mediated prostate cancer cell angiogenesis". In this paper, we identified the molecular mechanism by which vitamin D inhibits prostate cancer angiogenesis through IL-8, a key molecule involved in angiogenesis and thought to be involved in the prostate cancer progression. Via tissue microarray analyses, we found a strong correlation of IL-8 expression with prostate cancer disease progression, which strengthens the roles of IL-8 in the prostate cancer progression, thereby the inhibition of IL-8 by vitamin D strongly supports the chemotherapeutic effects of vitamin D in preventing prostate cancer progression.

Both of the publications serve as the basis for investigation of combination effects of 1α,25-vitamin D3 and COX-2 inhibitors for inhibition of prostate cancer progression.

The third paper was just accepted and will be published by *International Journal of Cancer*, 2008. The title of this paper is "A new prostate cancer therapeutic approach: combination of androgen ablation with COX-2 inhibitor". To explore the COX-2 roles in the prostate cancer progression, we applied *in vitro* cell studies to illustrate that inhibition of androgen/AR signal during the complete androgen blockage (CAB) therapy would consequently induce COX-2 expression. Suppression of COX-2 expression by NS-389 suppressed prostate cancer cell growth. The data was further confirmed by illustration of elevated COX-2 expressions in three pairs of prostate cancer patients before and after the CAB therapy. These findings provide a possible explanation how CAB therapy might fail and provide a strong rationale for therapeutic approach to battle prostate cancer using COX-2 inhibitor(s). Despite the cardiovascular complications associated with the use of the COX-2 inhibitors for prolonged periods of time, our studies suggest an urgent need for the development of more potent COX-2 inhibitors with fewer side effects.

Key Research Accomplishments:

Task 1: Evaluation of 1,25-VD suppression on prostate cancer cell angiogenesis.

- Examination of concentration of IL-8, an angiogenesis factor, in prostate cancer and 1,25-VD affect on its expression. Fig. 1 and 2 in Bao B.-Y., Yao J., and Lee, Y.-F., *Carcinogenesis*, 27: 1883-1893, 2006
- Examination of 1.25-VD effects on prostate cancer angiogenesis *in vitro* and its effect on IL-8.

  Fig. 3 in Bao B.-Y., Yao J., and Lee, Y.-F., *Carcinogenesis*, 27: 1883-1893, 2006.
- Determination of the molecular mechanisms how 1,25-VD suppresses prostate cancer cell angiogenesis Figs. 5a and 7 in Bao B.-Y., Yao J., and Lee, Y.-F., Carcinogenesis, 27: 1883-1893, 2006.

Task 2: Evaluation of the effects of 1,25-VD in combination with Docetaxel, one of most potent chemo reagent on progression of prostate cancer cells.

- Determination of the dose of 1,25-VD and Docetaxel on prostate cancer cell growth
  Fig. 1 in Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., *Cancer Letters* 247: 122-129, 2007.
- Examine the anti-tumor effects of these two reagents in combination.

Fig. 2 in Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., *Cancer Letters* 247: 122-129, 2007.

- Examine the apoptosis effects of these two reagents in combination.
  Fig. 3 in Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., *Cancer Letters* 247: 122-129, 2007.

Task 3: Evaluation of the underlying mechanism of the anti-tumor effect of combination of vitamin D and Docetaxel

- Determine the genes that are involved in the anti-tumor effects mediated by this drug combination.
  Fig. 4 in Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., *Cancer Letters* 247: 122-129, 2007.
- Determine the mechanism of combination of 1.25-VD with Docetaxel
  Fig. 5 in Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., *Cancer Letters* 247: 122-129, 2007.

Task 4: Investigation of the elevation of COX-2 expression *in vitro* and in prostate cancer patients with CAB therapy

- Examine the COX-2 expression in the prostate cancer cell lines with the modulation of androgen/AR signals.
  Figs. 1, 3, and 4 in Cai, Y, et al, *International Journal of Cancer*, 2008. (paper accepted)
- Identify NF-kB pathway as responsible for negatively regulation of COX-2 by androgen/AR signals
  Fig. 2 Cai, Y, et al, *International Journal of Cancer*, 2008. (paper accepted).
- Examination of COX-2 expression in the patients before and after CAB therapy
  Fig. 5 Cai, Y, et al, *International Journal of Cancer*, 2008. (paper accepted).

Task 5: Investigation of growth suppression effect by inhibition of COX2 activity in prostate cancer cells

- Suppression of prostate cancer cell growth, and colony formation by COX-2 inhibitor NS 389.
  Fig. 6. Cai, Y, et al, *International Journal of Cancer*, 2008. (paper accepted).

Reportable Outcomes:

- **Lee Y.-F**., Cai, Y., Li G., Liu S., Bao B-Y, Huang J, Hsu C.-L., and Chang C., A new prostate cancer therapeutic approach: combination of androgen ablation with COX-2 inhibitor *International J. of Cancer*, 2008 in press
- Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., "Docetaxel-induced growth inhibition and apoptosis in androgen independent prostate cancer cells are enhanced by 1a, 25-dihydroxyvitamin D3", *Cancer Letters* 247: 122-129, 2007.
- Bao B.-Y., Yao J., and Lee, Y.-F., "1α.25-dihydroxyvitamin D3 suppresses interleukin-8-mediated prostate cancer cell angiogenesis", *Carcinogenesis*, 27: 1883-1893, 2006.

Conclusion:

The clinical use of COX-2 inhibitors has recently become controversial due to cardiovascular complications associated with the use of COX-2 inhibitor for prolonged periods of time. To explore the COX-2 roles in the prostate cancer disease progression, we applied *in vitro* model and found that the blockage of androgen signals such as in CAB therapy would consequently result in elevated COX-2 expression. Clinical samples from the prostate cancer patients further confirm the negative correlation between COX-2 and androgen/AR signals. And suppression of COX2 expression would then have beneficial effects by suppression prostate cancer cell growth. Therefore our studies suggest an urgent a need for development of more potent COX-2 inhibitors with fewer side effects.

In addition to combination with COX-2 inhibitor, vitamin D-based combination therapy, such as with docetaxel, was also then developed in our study. Docetaxel is the only treatment shown to improve overall survival in hormonal refractory prostate cancer patients and the combination of docetaxel with 1,25-VD have been proven to decrease PSA level in the patients.

As demonstrated in our progress report above, through the publication of three papers related to the vitamin D anti-prostate cancer angiogenesis, development of vitamin D based therapy strategy as well as investigation of COX-2 roles in prostate cancer progression, we conclude that vitamin D and COX-2 inhibitors are very powerful chemotherapy agents to halt prostate cancer progression. Development of more potent agents with fewer side effects that are associated with both agents: hypercalcimia and cardiovascular disease, would be of interest for the future direction of this project.

References and Appendices: (These are the same as the reportable outcomes.)

- 1. Lee Y.-F., Cai, Y., Li G., Liu S., Bao B-Y, Huang J, Hsu C.-L., and Chang C., A new prostate cancer therapeutic approach: combination of androgen ablation with COX-2 inhibitor International J. of Cancer, 2008 in press

- 2. Ting H.-J., Hsu J., Bao B.-Y, and Lee, Y.-F., "Docetaxel-induced growth inhibition and apoptosis in androgen independent prostate cancer cells are enhanced by 1a, 25-dihydroxyvitamin D3", *Cancer Letters* 247: 122-129, 2007.

- 3. Bao B.-Y., Yao J., and Lee, Y.-F., "1α, 25-dihydroxyvitamin D3 suppresses interleukin-8-mediated prostate cancer cell angiogenesis." *Carcinogenesis*, 27: 1883-1893, 2006.

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A new prostate cancer therapeutic approach: combination of androgen ablation with COX-2 inhibitor

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Key Words:	Prostate cancer, cyclooxygenase 2 , combined androgen blockade

A new prostate cancer therapeutic approach: combination of androgen ablation with COX-2 inhibitor

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Key Words: Prostate cancer, combined androgen blockade, cyclooxygenase 2,

Abbreviations: CAB, combined androgen blockade; PG, prostaglandins; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; NSAIDS, non-steroidal anti-inflammatory drugs; AR, androgen receptor; DHT, 5α-dihydrotestosterone; HF, hydroxyflutamide

Running title: COX-2 roles in prostate cancer progression

Abstract

Prostate cancer is initially responsive to hormonal therapy, but cancers inevitably progress in an androgen-independent fashion with virtually all tumors evolving into more aggressive androgen refractory disease. Immunohistological comparisons of cyclooxygenase 2 (COX-2) expressions in three pairs of prostate cancer patients before and after the combined androgen blockade (CAB) therapy show elevated COX-2 expressions. This observation from clinical specimens is further supported by *in vitro* laboratory data using human prostate cancer cells in which the antiandrogen hydroxyflutamide (HF) induced COX-2 expression, and androgen suppressed COX-2 expression. By applying knockdown and overexpression strategies to modulate AR expression in prostate cancer cells, we confirmed that androgen/AR signal suppressed, and HF induced COX-2 expression at both protein and mRNA levels. COX-2 promoter reporter assay indicated that the suppression of COX-2 by androgen/AR is at the transcriptional level via modulation of NF-κB signals. Treatment of LNCaP and LAPC4 cells with 1 μM HF in the presence of 1 nM DHT, which mimics the CAB therapy condition, promotes cell growth, and this growth induction can be suppressed via adding the COX-2 specific inhibitor, NS398. This suggests that HF promoted prostate cancer cell growth is COX-2 dependent and this HF-COX-2 activation pathway can account for one reason of CAB therapy failure. Together, these findings provide a possible explanation how CAB with antiandrogen HF therapy might fail and provide a potential new therapeutic approach to battle prostate cancer via combination of CAB therapy with COX-2 inhibitor(s).

Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer death among men in the United States [1, 2]. Ever since Charles Huggins and his colleagues [3] first demonstrated that androgen ablation could control prostate cancer growth, combined androgen blockade (CAB) therapy, which is based on suppression of androgen signaling, has played a major role in the management of this disease. CAB strategy usually utilizes luteinizing hormone releasing hormone (LHRH) agonists and antiandrogens to suppress the circulating androgen level and block the function of the androgen receptor (AR) [4]. Although 85 to 90 percent of advanced prostate cancer patients respond to CAB therapy, it is not curative, with the response lasting for less than 2 years. Then, virtually all cancers inevitably progress into an androgen-independent state [5, 6]. The mechanisms by which prostate cancer cells survive after CAB therapy remain unclear.

Derived from arachidonic acid, prostaglandins (PGs) are a family of biologically potent fatty acids, which regulate various pathophysiological processes such as inflammatory reaction, gastro-intestinal cytoprotection and ulceration, and hemostasis [7]. Cyclooxygenase 1 and 2 (COX-1 and -2) are the rate-limiting enzymes in the PG synthesis [8]. COX-1 is constitutively expressed in most tissues, whereas COX-2 is generally expressed at very low levels, but can be induced by growth factors, tumor promoters, and pro-inflammatory cytokines [9, 10]. Compelling data from basic and clinical research suggest that overexpression of COX-2 has been implicated in the initiation and progression of a large number of human cancers [11-15]. The application of non-steroidal anti-inflammatory drugs (NSAIDs), as well as COX-2 specific inhibitors in cancer clinical practice, has drawn much attention in the past few years as inhibition of COX-2 might provide an effective strategy in the prevention and treatment of cancer.

The role of COX-2 in prostate cancer has been controversial. Several studies showed greater immunostaining of COX-2 in prostate cancer as compared with benign prostatic tissue [16, 17], and the intensity of immunostaining was positively correlated with high tumor grade (Gleason score 8 and 9 vs. 5 to 7) [16]. In contrast, another study

found COX-2 expression was not up-regulated in established prostate cancer and high grade PIN, as compared with adjacent normal prostate tissue [18]. The controversy requires further studies to elucidate the roles of COX-2 in prostate cancer. However, previous studies have never addressed the correlation between COX-2 and CAB therapy and their roles in prostate cancer progression. In our current study, we found 1 nM DHT plus 1 μM HF enhanced COX-2 expression in prostate cancer patients' samples as well as in LNCaP cells, and androgen alone inhibits COX-2 expression in LNCaP cells through mediating the NF-κB pathway. NS398, a COX-2 specific inhibitor, significantly blocked LNCaP cell proliferation stimulated by androgen ablation therapy. These data suggest that overexpression of COX-2 after androgen ablation might be involved in the progression from an androgen-dependent to an androgen-independent phenotype and combination of androgen ablation with anti-COX-2 inhibitors could become a new potential approach to battle the prostate cancer.

Materials and methods

Reagents and antibodies

Primary antibodies to COX-2 peptides were purchased from Cayman Biochemical Co. (Rabbit polyclonal IgGs; Ann Arbor, MI). The specificity of this COX-2 antibody was verified by other reports by COX-2 blocking peptide [19-21]. COX-2–selective inhibitor NS398 was purchased from Cayman BioChemical Co. (Ann Arbor, MI).

Immunohistochemica (IHC) staining

COX-2 IHC staining was performed using the ABC kit (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame). Briefly, sections were rinsed with methyl alcohol–hydrogen peroxide and then microwaved in citrate buffer (pH 6.0) to induce epitope retrieval. Diluted COX-2 primary antibody (1:100) was incubated on slides at 4^0C overnight and then incubated with biotinylated secondary antibody at room temperature. For localization, avidin–biotin complex was applied at room temperature for 30 mins followed by 3,30-diaminobenzidine tetrahydrochloride as the choursomagen. Slides were counterstained with Mayer hematoxylin. As a negative control for nonspecific staining, COX-2 blocking peptide was added to the diluted COX-2 antibody at a final concentration of 10 mg/ml and incubated for 1 hr at room temperature before the application of the COX-2 antibody to the slides. Then the manual IHC staining was performed as described above [21].

Cell culture and plasmids

LNCaP, PC-3, and COS-1 cell lines were purchased from the American Type Culture Collection (Rockville, MD). PC-3(AR-2) cell was a gift from Dr. Theodore J. Brown (Department of Zoology, The University of Toronto, Toronto, Ontario, Canada). LAPC-4 cell line is a gift from Dr. Sawyers (Department of Medicine, Jonsson Comprehensive Cancer Center, University of California at Los Angeles, CA) and maintained in Iscove's modified Dubecco's medium. The LNCaP, PC-3, PC-3(AR-2), CWR22R, and CWR22-AR↓ cell lines were maintained in RPMI medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (Gibco-BRL, Grand Island, NY), and COS-1 monkey kidney cells were maintained in phenol red-free Dulbecco's

modified Eagle's medium (DMEM) containing 10% FBS and penicillin/streptomycin all at 5% CO_2, and 37^0C. The plasmids pGL3-COX-2 promoter and its serial deletion mutant luciferase (*Luc*) reporter gene were gifts from Dr. Rama Natarajan (Gonda Diabetes Center, Beckman Research Institute of City of Hope, Duarte, CA).

Transfections and reporter gene assays

Transfections were performed by using SuperFect according to the manufacturer's suggested procedures (Qiagen) [22]. After transfection, cells were treated with charcoal-dextrans-stripped FBS medium containing either ethanol or ligands for 24 hr. Cell lysates were prepared, and the luciferase (Luc) activity was normalized for transfection efficiency using pRL-CMV as an internal control. Luc assays were performed using the dual-luciferase reporter system (Promega, Madison, WI). Briefly, 1-4 x 10^4 cells were plated on 12-well plates 24 hrs before adding the precipitation mix containing Luc reporter genes. The medium was changed 24 hrs after transfection, and the cells were treated with 1 nM DHT for 24 hrs, followed by various treatments for another 16 hrs. The cells were then harvested, and whole cell extracts were used for the Luc assay. Luc activity was determined using a Dual-Luciferase Reporter Assay System (Promega) and measured with a luminometer.

RT-PCR and real-time PCR

Total RNA was extracted from prostate cancer cells using Trizol (Invitrogen). We carried out reverse transcription with the Super Script II kit (Invitrogen) and PCR amplifications with SYBR Green PCR Master Mix on an iCycler IQ multi-color real-time PCR detection system (Bio-Rad). The COX-2 primer pairs were 5'-CCCTGAGCATCTACGGTTTG-3' and 5'-CATCGCATACTCTGTTGTGTTC-3'. The AR primer pairs were 5'-CCTGGCTTCCGCAACTTACAC-3' and 5'-GGACTTGTGCGTGCGGTACTCA-3'. The normalization control used was β-actin, and the primers were 5'-CAGCTCTGGAGAAACTGCTG-3'; and 5'-GTGTACTCAGTCTCCAC AGA-3'. ΔCT values were calculated by subtracting the threshold (CT) value from the corresponding β-actin CT (internal control) value from each time point. Then ΔΔCT values were calculated by subtracting the ΔCT value of

untreated controls from the ΔCT value of treated samples. The absence of non-specific amplification products was confirmed by agarose gel electrophoresis.

Western blot analysis

Whole cell lysates were made by a standard method, and protein concentrations were measured with the BCA protein reagent (Pierce Chemical Co., Rockford, IL). Approximately 100 µg of protein/lane was loaded and run on a 10% polyacrylamide gel with a Tris/glycine running buffer system and then transferred onto a polyvinylidene difluoride membrane. The blots were probed with primary AR and COX-2 antibodies with dilutions of 1:250 to 1:1,000 and incubated at room temperature for 2 hrs. The secondary antibody [rabbit antigoat IgG, 1:1,000 dilution (Santa Cruz Biotechnology, Inc.) or rabbit antimouse IgG, 1:1,000 dilution (Pierce Chemical Co., Rockford, IL)] was used at room temperature for 1 hr. Immunoblot analysis was performed with horseradish peroxidase-conjugated antirabbit and antimouse IgG antibodies using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences).

Cell proliferation assay in vitro

Cell medium was replenished and cell proliferation was determined by MTT assay (Sigma). Serum-free medium containing MTT (0.5 mg/ml) was added into each well. After 4 hrs incubation at 37^{o}C, the stop solution was added to solubilize the formazan product and the absorbance was recorded. Data are expressed as the mean ± S.D. of triplicate samples.

Colony formation assay

LNCaP or LAPC4 cells that were under various treatments for 24 hours, then cells were trypsinized, counted, and seeded at 500 cells/dish in 60-mm tissue culture dishes. Cells were fed with fresh growth media every 4–5 days for 2–3 weeks until the colonies were well formed. Crystal violate stain was used to visualize the colonies.

Immunofluorescence staining

LNCaP cells were seeded on two-well Lab Tek Chamber slides (Nalge) in RPMI 1640 medium containing 10% FBS. Cells were cultured with 10% charcoal-dextran-stripped fetal bovine serum for 24 hrs, and then were treated with 1 nM DHT, 10 nM

DHT, 1 μM HF, 1 nM DHT plus 1 μM HF, or vehicle for 24 hrs. Then cells were fixed with fixation solution (3% paraformaldehyde and 10% sucrose in phosphate-buffered saline) for 20 mins on ice, followed by permeabilization with methanol for 10 mins at -20^{0}C. Slides were washed and blocked with 2% bovine serum albumin in phosphate-buffered saline for 15 mins at room temperature. Then the cells were stained with 1 μg/ml of rabbit polyclonal anti-COX-2 antibody at room temperature for 1 hr. After the first antibody incubation, cells were washed and incubated with Texas Red-conjugated goat anti-rabbit IgG. Stained slides were coverslipped and visualized with a fluorescence microscope.

Results

Androgen/androgen receptor suppressed COX-2 expression involves NF-κB binding signals

To investigate the roles of COX-2 expression in the prostate cancer progression during CAB therapy, we examined the DHT effect on COX-2 expression in the androgen-sensitive prostate cancer cell line LNCaP. The basal level of COX-2 mRNA in LNCaP cells was almost undetectable by semi-quantitative PCR analysis, similar to other studies [23]. Therefore, real-time PCR assays were used to compare the relative expression level of COX-2 upon the DHT treatment. The results showed that COX-2 mRNA expression (Fig. 1*a*), as well as protein amounts (Fig. 1*b*), were decreased when the androgen/AR signals were activated by both 1 and 10 nM DHT treatment in LNCaP cells.

To study if AR is involved in this DHT-mediated COX-2 suppression, we applied both knockdown and overexpression of AR strategies. As shown in Fig. 1*c*, AR expression was increased in PC-3 stably transfected functional AR cells (PC-3AR2) and reduced in CWR22R-AR↓ cells in which AR was knocked down by homologous recombinant gene targeting [24], and both cells were maintained in 10% FBS culture medium to sustain androgen level. Consistent with the data shown in Fig. 1*a* and *b*, COX-2 mRNA expression was reduced when AR expression increased in PC-3AR2 and COX-2 mRNA expression was increased when AR expression decreased in CWR22R-AR↓ cells (Fig. 1*c*). The same tendency was further confirmed in the COX-2 protein levels (Fig. 1*d*) in those two prostate cancer cell lines.

DHT/AR suppresses COX-2 expression at both RNA and protein levels indicating that regulation of COX-2 by DHT/AR is at the transcriptional level. To confirm this, DHT/AR effect on COX-2 5′ promoter activity was studied. As shown in Fig. 2*a*, the activity of COX-2 5′ promoter containing *Luc* reporter was suppressed in the presence of 1 nM or 10 nM DHT, while TPA, as a positive control, can activate COX-2 promoter activity. As sequence analysis of the COX-2 gene 5′ promoter region found no classical androgen response element, we hypothesized that DHT/AR suppresses COX-2 gene expression through modulation of NF-κB, a known DHT/AR regulated gene [25] and upstream modulator for COX-2 gene [26]. To identify the DHT/AR corresponding region,

we serially deleted COX-2 5′ promoter into three regions: #1, #2 and #3 constructs which contains one, two, and no NF-κB binding sites, respectively. As shown in Fig. 2*a*, we found that DHT at 1 and 10 nM, suppressed COX-2 gene expression, and 1 nM TPA, an NF-κB inducer, promoted COX-2 gene expression. However, this suppression of COX-2 gene expression was lost in the #3 COX-2 reporter construct in which the NF-κB binding site was deleted (Fig. 2*b*), suggesting that DHT/AR transcriptionally suppresses COX-2 gene expression through regulating the NF-κB signal pathway.

CAB with HF therapy stimulates COX-2 gene activity in prostate cancer LNCaP cells

The above data suggested that DHT/AR could suppress COX-2 gene expression at the transcriptional level; therefore blockage of androgens by CAB therapy might then release the suppression of COX-2 gene expression in prostate cancer cells and result in elevated COX-2 expression. According to the report by Dr. Mohler the mean total prostate DHT concentration before *vs* after CAB therapy are 2-10 nM DHT *vs* 1-3 nM DHT, respectively [27]. To mimic the clinical CAB therapy scenario, we treated LNCaP cells with 1 nM DHT and 1 μM HF, and found that COX-2 gene expressions were increased at both mRNA (Fig. 3*a*) and protein levels (Fig 3*b*). Similarly, the COX-2 5′ promoter activity was also stimulated by the combination of DHT and HF (Fig. 3*c*). WE also found that 1 μM HF alone and in combination with DHT induced COX-2 expression, while DHT alone suppressed COX-2 expression suggesting that HF might not only induce COX-2 expression via antagonist to androgen/AR-suppressive COX-2 effect, but also via androgen/AR-independent pathways.

COX-2 is a key enzyme involved in the production of prostaglandins (PG), which requires the condensation of COX-2 into the nuclear envelope [28]. To test if increased COX-2 gene expression after CAB with HF therapy could also result in increased functional COX-2 within the nucleus, we performed immunofluorescence staining of COX-2 in LNCaP cells. As shown in Fig. 4, COX-2 proteins were mainly located in the nucleus when cells were treated with 1 μM HF or vehicle control. In contrast, COX-2 proteins were retained in the cytoplasm when cells were treated with 1 nM DHT, the androgen concentration after CAB therapy, and HF+DHT treatment can reverse the DHT-induced COX-2 cytoplasmic retention where COX-2 was located in the nucleus and

its expression was restored to the level similar to vehicle-treated cells. Together, these data clearly demonstrate that CAB with HF therapy would increase COX-2 gene expression as well as promote its nuclear translocation, thus resulted in increasing functional nuclear COX-2 protein in prostate cancer cells.

Treatment with CAB and HF therapy results in the increased COX-2 expression in prostate cancer patients

To further confirm the above *in vitro* findings, we examined COX-2 protein expression in clinical specimens from prostate cancer patients before and after CAB therapy. Three pairs of prostate tumor specimens obtained from patients before and after CAB therapy were subjected to the COX-2 immunostaining. As shown in Fig. 5, very weak COX-2 expression was detected mainly in the cytoplasm of luminal epithelial cells in all three specimens before androgen ablation therapy. In contrast, an enhanced and strong COX-2 expression was detected in both the cytoplasm and nuclei of epithelial cells in all specimens from the same patients after CAB therapy. Therefore, these observations from clinical specimens support our above findings in prostate cancer cell lines.

A new potential therapy via combination of CAB with HF therapy and anti-COX-2 inhibitor

Our studies in both *in vitro* prostate cancer cells and *in vivo* clinical data suggested that CAB with HF therapy, the currently used therapy to treat prostate cancer, might result in the increased COX-2 expression in the prostate tumor. COX-2 has been implicated to play important roles in the cancer progression and invasion [29, 30], therefore, we were interested to know the cellular response to elevated COX-2 expression. Colony formation assays were used to examine the cell response to treatments in LNCaP and LAPC4 cells. As shown in Fig 6*a*, a treatment with 1 nM DHT alone or 1 μM HF alone, and a combination of both, mimicking prostate patients' serum level after CAB with HF therapy, promotes cell growth (Fig. 6*a*). Importantly, treatment with NS398, an COX-2 inhibitor, results in the significant suppression of DHT/HF-induced prostate cancer cell growth. As a control, cell growth was slightly inhibited when LNCaP cells were treated

with NS398 alone. Note that LAPC4 cells, which contain wild type AR respond better to treatment than LNCaP cells, which contain mutated AR. To further confirm these results, we applied the MTT assay to examine LNCaP and LAPC4 cell growth upon the treatments. As shown in Fig. 6*b*, DHT, HF, and DHT+HF can promote, and NS398 alone suppress, cell growth. The combination of DHT and HF promoted cell growth significantly, starting at day 4, and adding NS398 reverses the DHT+HF induced cell growth significantly. Together, the data from two AR positive prostate cancer cell lines suggested that the elevated COX-2 expression due to CAB with HF therapy stimulates cell growth, which might contribute to the failure of CAB therapy. And applying COX-2 inhibitor(s), such as NS398, might be able to reverse this adverse effect derived from CAB therapy.

Discussion

Prostate cancer is one of the most commonly occurring cancers with a high mortality rate among American men. In patients with advanced prostate cancer, CAB therapy using currently available antiandrogens is a standard treatment option, but it almost results in the emergence of androgen-independent disease and eventual mortality. The precise molecular events that lead from androgen-sensitive prostate cancer to androgen-refractory prostate cancer are, therefore, critical for decoding prostate cancer progression as well as developing specific therapies that can interfere with these pathways to stop disease progression.

It has been proposed that there are many pathways involved in the development of androgen-independent prostate cancer, via an AR-dependent or -independent pathway [31-33]. The mechanisms that involve the AR directly include mutations in, or amplification of, the AR gene in a manner that allows the AR to respond to low doses of androgens, other steroids, or antiandrogens [34, 35]. In a less direct manner, AR coactivators might increase the sensitivity of the AR to androgens and even other nonandrogenic substances through a number of mechanisms [34, 36] Additional indirect mechanisms that do not result from mutations of the AR might involve activation of the AR by peptide growth factors or cytokines [37, 38]. The AR-independent pathways that bypass AR involve neuroendocrine differentiation of prostate cancer cells [39], deregulation of apoptotic genes [40], and unknown

mechanisms related to down-regulation of AR [41] or the HF activated MAP kinase pathway [42].

COX-2 has been shown to be involved in prostate cancer, several other human cancers, and inflammatory diseases. Therefore, the potential use of NSAIDs, well known COX inhibitors, as chemopreventive or therapeutic agents for a variety of malignancies, including prostate cancer, is being intensely investigated. Potential mechanistic roles of COX-2 in tumorigenesis and tumor progression include decreased apoptosis and immune surveillance, increased angiogenesis, and tumor invasiveness [29, 43]. There have been strong preclinical, epidemiologic, and clinical data supporting an association between NSAID use and a reduced incidence of and mortality from cancer [44]. Based on results from laboratory and clinical studies, it has been suggested that inhibition of COX-2 might be a useful chemopreventive/therapeutic option for prostate cancer [45]. Our studies demonstrated that androgen/AR suppressed, and that 1 μM HF plus 1 nM DHT, which mimics the clinical CAB therapy condition, enhanced COX-2 expression. These results strongly suggest that CAB therapy might induce COX-2 expression, an unwanted CAB side effect, which might then lead to the transition of androgen-independent disease, as well as cancer progression. Our *in vitro* proliferation assay further confirmed that co-treatment with COX-2 inhibitor NS398 significantly suppressed 1 μM HF/1 nM DHT stimulated LNCaP cell growth. Therefore, a combination of a specific COX-2 inhibitor and CAB therapy, which might delay progression of prostate cancer from an androgen-dependent to an androgen-independent stage, could be a useful therapeutic strategy in prostate cancer.

The clinical use of COX-2 inhibitors has recently become controversial because of the cardiovascular complications associated with the use of high doses of COX-2–selective NSAIDs for prolonged periods of time [46]. In comparison with the COX-2–selective inhibitors, the use of a nonselective NSAID, such as naproxen, has been shown to be associated with decreased cardiovascular adverse effects [47]. As shown by our study, a growth inhibition was seen with a COX-2 inhibitor while cells were stimulated by CAB therapy; therefore, it is worthy to apply the clinical utility of a nonselective NSAID, such

as naproxen, to eliminate the cancer cell growth induced by CAB therapy, thereby restraining prostate cancer progression.

In conclusion, androgen and its receptor play an inhibitory role in COX-2 expression in prostate cancer cells. COX-2 overexpression in prostate cancer is due to androgen ablation, and might result in the failure of CAB therapy. Our findings suggest that cotreatment with COX-2 inhibitor(s) could diminish prostate cancer progression induced by androgen ablation therapy.

Acknowledgments

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Figure Legends:

FIGURE 1 -Androgen/androgen receptor suppresses COX-2 expression. (*a*) The mRNA level of COX-2 was quantified with real-time PCR in LNCaP cells treated with androgen (1 and 10 nM of DHT). (*b*) Western Blot was performed to analyze COX-2 protein in LNCaP cells. 5×10^5 LNCaP cells were seeded on 60 mm Falcon dishes with RPMI medium-10% FBS and penicillin/streptomycin. Cells were treated with ethanol, 1 nM DHT or 10 nM DHT for 2 days. 100 µg of total protein from LNCaP cells was applied onto an 8% sodium dodecylsulfate-polyacrylamide gel and subjected to electrophoresis followed by electrotransfer to a membrane. Immobilized protein was detected with anti-COX-2 antibody, (*c*) and (*d*) endogenous levels of mRNA and protein of COX-2 and AR in CWR22R, CWR22R-AR↓, PC-3AR2, and PC-3 cells. Real-time PCR and Western Blot analyses were performed.

FIGURE 2 - Androgenic regulation of COX-2 is mediated by NF-κB binding sites in the COX-2 promoter. (*a*) Effects of 1 nM and 10 nM DHT on COX-2 transactivation in LNCaP cells. 2×10^4 LNCaP cells were seeded onto 12-well plates. Cells were transfected with luciferase reporter plasmid, pGL3-COX-2, and pT plasmid as an internal control. After 4 hrs, the medium waschanged to CD medium and incubated overnight. Then cells were treated with ethanol, 1 nM DHT or 10 nM DHT. After 24 hours, cells were lysed with luciferase lysis buffer, and luciferase activity of the cell lysates was determined using a Dual-luciferase Reporter Assay System and measured with a luminometer. (*b*) Diagram of serial deletion mutants (#1, #2, and #3) of the human COX-2 promoter (from -1,437 and +127 bp relative to the start of transcription (+1 bp)) luciferase plasmid (upper panel). Three deletion mutants constructs: #1 (-1437 to +127) construct contains two NF-κB binding sites, #2 (-360 to +127) construct contains one NF-κB binding sites, and #3 (-218 to +127) construct contains no NF-κB binding site were transfected into LNCaP cells, then cells were treated with 1 nM DHT 24 hr after transfection. The Luc reporter assay was performed to determine the DHT-mediated COX-2 transactivation activity.

FIGURE 3 - CAB with HF therapy stimulates COX-2 expression. (*a*) The expression level of COX-2 mRNA in LNCaP cells treated with 1 μM HF or 1nM DHT alone, or in combination with both reagents with real-time PCR. (*b*) Western Blot was performed to analyze COX-2 protein in LNCaP cells that were treated with ethanol, 1 μM HF or 1 nM DHT, or in combination with both reagents for 2 days. C, Luciferase assay was used to determine effects of 1 μM HF or 1 nM DHT alone, or in combination with both regents on COX-2 promoter transactivation activity.

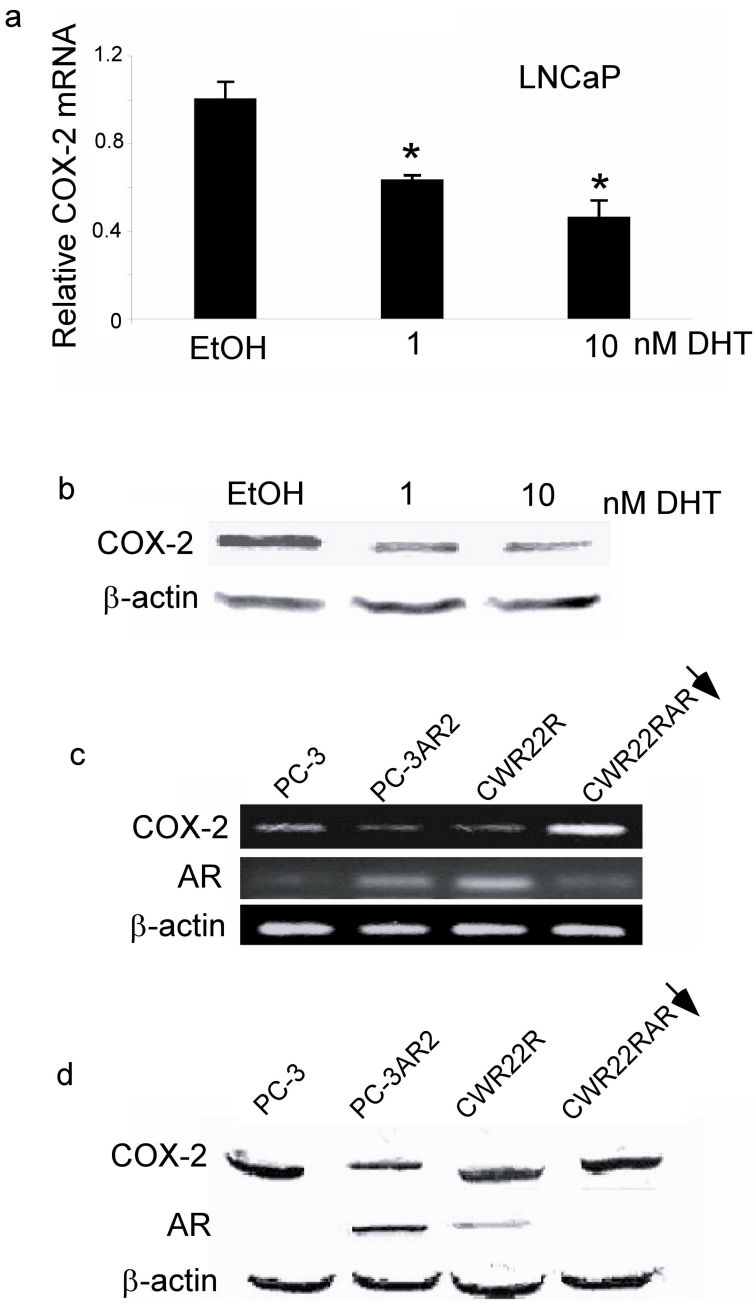
FIGURE 4 - CAB with HF therapy promotes COX-2 nuclear translocation. LNCaP cells were treated with vehicle, 1 nM DHT, 1 μM HF, and 1 nM DHT plus 1 μM HF, and COX-2 cellular localization was determined by Immunofluorescence staining.

FIGURE 5 - COX-2 expression in prostate cancer specimens before and after CAB therapy as detected by immunohistochemistry. Very weak immunoreactivity in prostate cancer patient specimens before CAB therapy was observed in the cytoplasm of epithelial cells (left panel) and strong immunoreactivity was observed in both cytoplasm and nuclei of the epithelial cells in specimens from the same patients after CAB therapy (right panel). Pictures were taken under x100 or x400 magnification.

FIGURE 6 - NS398 suppresses DHT + HF-induced prostate cancer cells growth. (*a*) LNCaP and LAPC4 cells were treated with 1 nM DHT, 1 μM HF, 25 μM NS398, and in various combinations for 24 hrs, then cells were trypsinized, counted, and seeded at 500 cells/dish in 60-mm tissue culture dish. Cells were fed with fresh growth media every 4–5 days for 2–3 weeks until the colonies were well formed. Crystal violet stain was used to visualize the colonies. (*b*) 5×10^4 LNCaP cells and LAPC4 were seeded onto 12-well plates for 24 hrs in cultured medium. Cells were then changed to culture medium with 10% CD FBS that contained ethanol, 1 nM DHT or 1 μM HF, or the combination of 1 nM DHT and 1 μM HF with 25 μM NS398. Cells were harvested every two days to

determine cell proliferation rate by MTT assay. **, P>0.01; *, p>0.05 statistics were examined by student t-test.

Fig. 1 Cai et al



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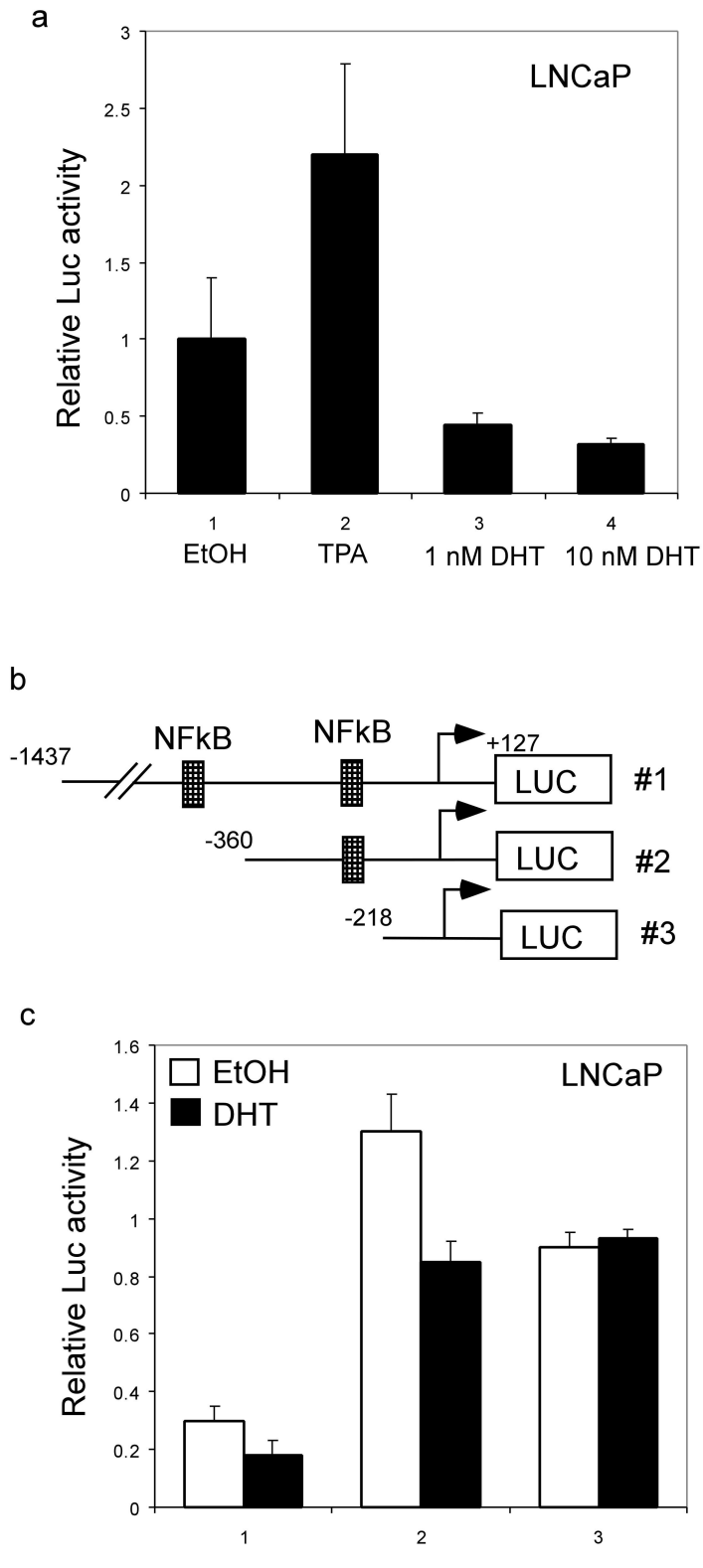

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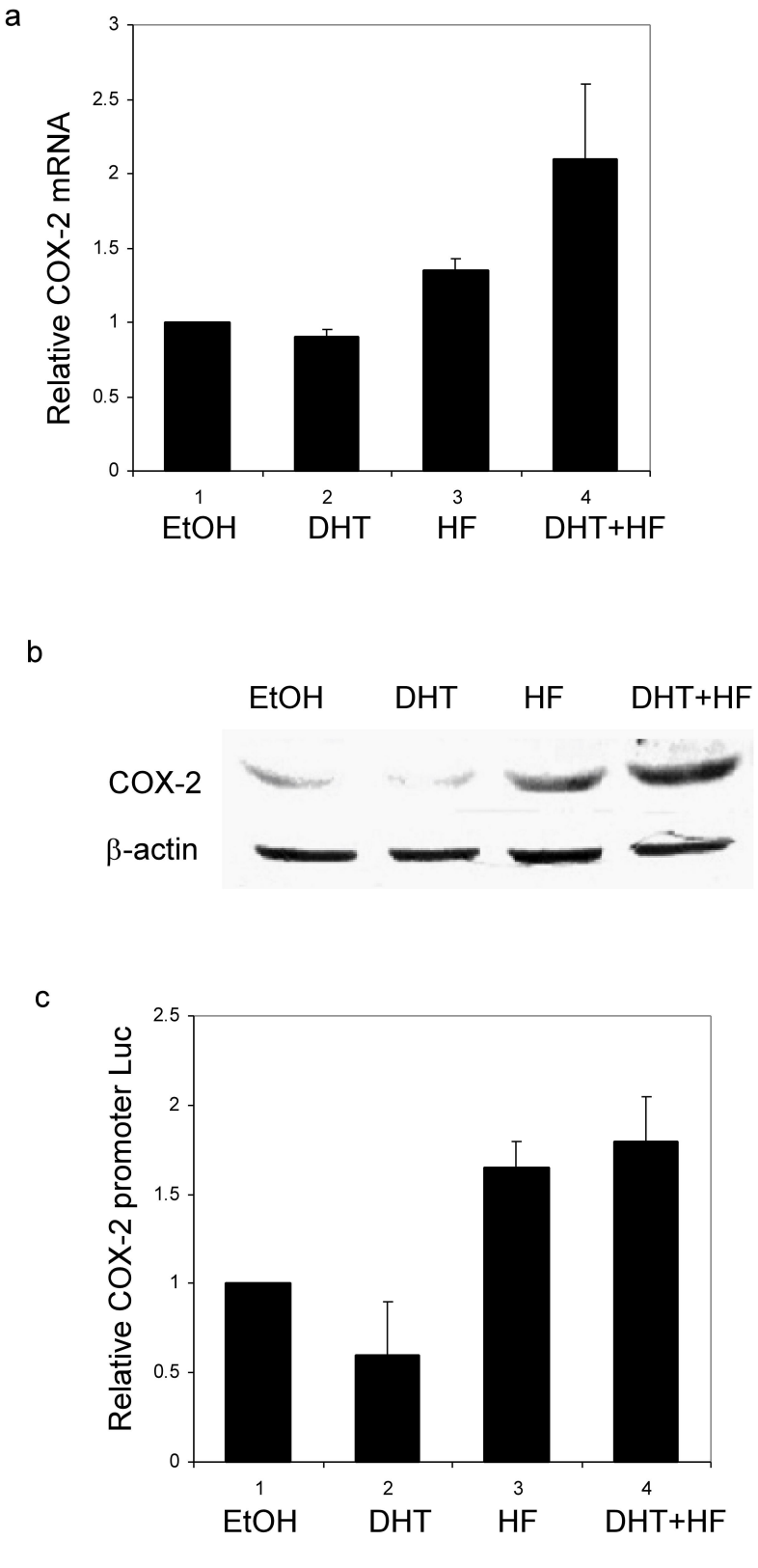

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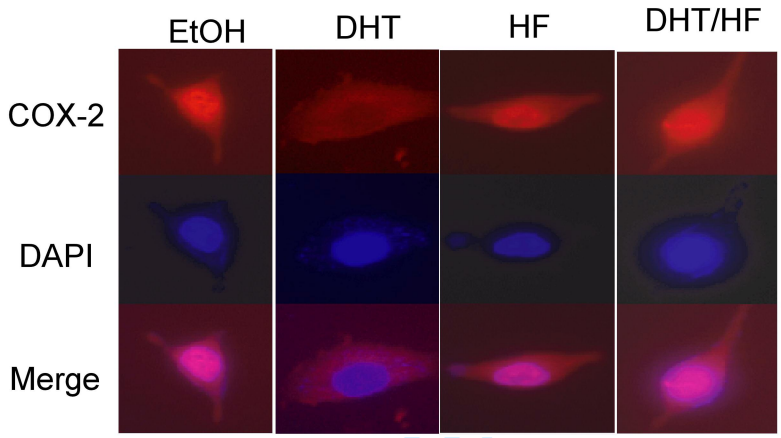

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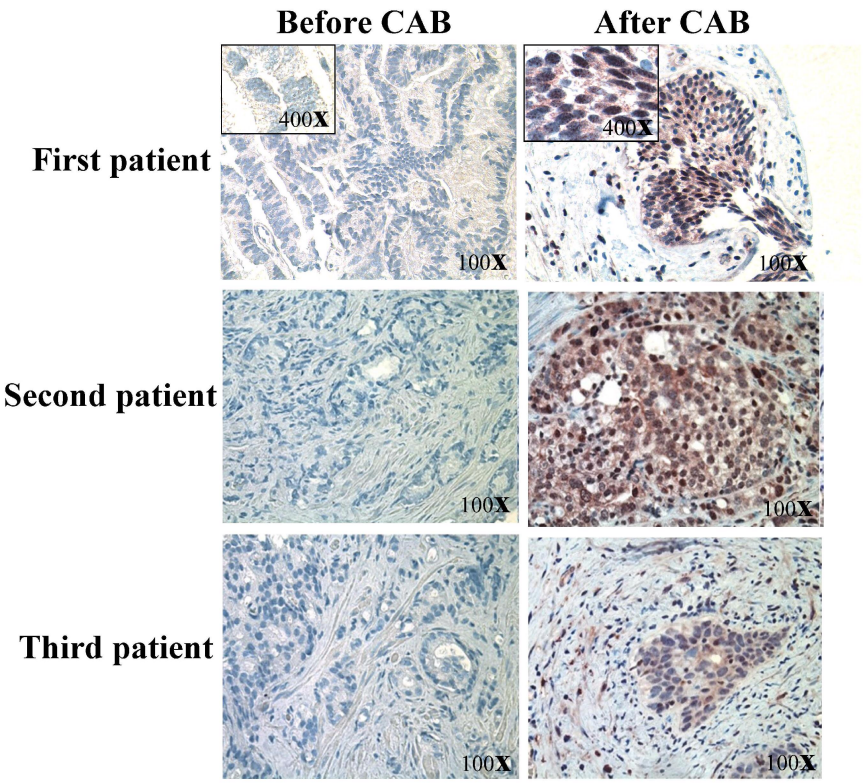

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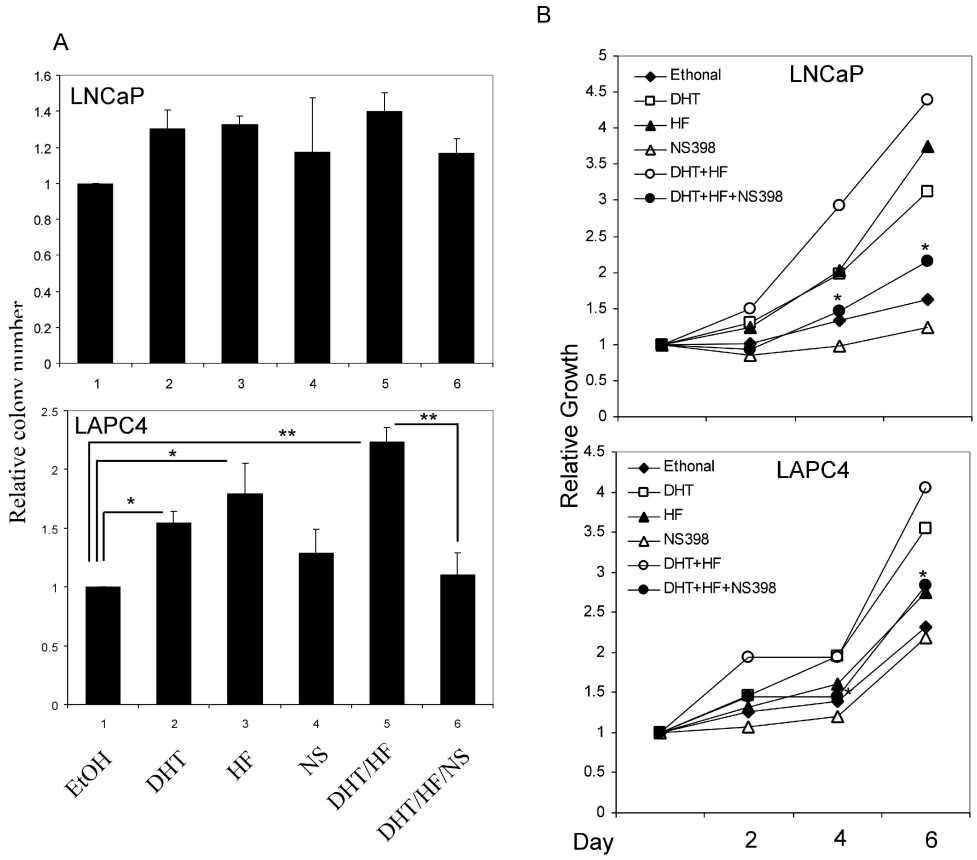

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Docetaxel-induced growth inhibition and apoptosis in androgen independent prostate cancer cells are enhanced by 1α,25-dihydroxyvitamin D_3

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Abstract

Pre-treatment with high-dose 1α,25-dihydroxyvitamin D_3 (1,25-VD) enhanced the antitumor activity of docetaxel in the androgen-independent prostate cancer cell line, PC-3. The effect manifested as an increasing population of apoptotic cells and amount of pro-apoptotic protein, Bax, under combined treatment compared with single treatment of either 1,25-VD or docetaxel alone. We further demonstrated that pre-treatment with 1,25-VD reduced the expression of multidrug resistance-associated protein-1 at both the mRNA and protein levels. This suggests pre-treatment with 1,25-VD can potentiate cytotoxicity of docetaxel in PC-3 due to 1,25-VD reducing multidrug resistance-associated protein-1 expression.

Keywords: Prostate cancer; Docetaxel; 1α,25-Vitamin D_3; Growth inhibition; Multidrug resistance proteins

1. Introduction

Chemotherapy remains one of the major options for effective treatment for hormone refractory prostate cancer (HRPC). In clinical trials, docetaxel (DX) (Taxotere; Aventis Pharmaceuticals, Inc, Bridgewater, NJ), a semi-synthetic taxane, effectively reduced prostate specific antigen (PSA) levels and improved symptoms in patients with HRPC [1,2]. Treatment with DX in combination with the active form of vitamin D has shown promising results in PSA response, time to progression, and survival in HRPC patients [3]. It is therefore, of interest to investigate the mechanism of these drug interactions for future therapeutic design.

It is widely believed that DX, similar to other members of the taxane family, binds to β-tubulin, inhibits microtubule depolymerization, and impairs mitosis hence retarding cell cycle progression in the G2/M phase [4]. During the G2/M phase arrest of cancer cells induced by DX treatment, Bcl-2 phosphorylation occurs [5]. Phosphorylation of Bcl-2 decreases its binding to the proapoptotic protein, Bax, which is released, translocates, and inserts into the mitochondrial membrane releasing cytochrome *c*, leading to apoptosis [6].

Abbreviations: 1,25-VD, 1α,25-dihydroxyvitamin D_3; DX, Docetaxel; HRPC, hormone refractory prostate cancer; PSA, prostate specific antigen; PCa, prostate cancer; VDR, vitamin D receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; EtOH, ethanol; 5-CF, 5-carboxylfluorescein; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; Q-PCR, quantitative PCR.

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The antiproliferative effect of 1α,25-dihydroxyvitamin D_3 (1,25-VD), the active form of vitamin D, has been reported in several types of cancers including prostate cancer (PCa). Both apoptosis and G0/G1 accumulation were observed in 1,25-VD treated PCa cells. 1,25-VD triggered apoptosis in LNCaP and ALVA-31 is accompanied by decreased expression of several antiapoptotic proteins, Bcl-2, Bcl-X_L, Mcl-1, BAG1L, XIAP, cIAP1, and cIAP2 [7]. Overexpression of Bcl-2 blocked 1,25-VD induced apoptosis in LNCaP cells, indicating the importance of Bcl-2 in the antiproliferative effect of 1,25-VD [8]. Induction of $p21^{WAF1/CIP1}$ expression, Rb hypophosphorylation, and CDK2 activity reduction result in G0/G1 accumulation in 1,25-VD treated LNCaP cells [9]. The fact that stable expression of $p21^{WAF1/CIP1}$ antisense or loss of $p21^{WAF1/CIP1}$ expression in certain PCa cell lines both abolished the antiproliferative effect of 1-25-VD indicates the increased expression of $p21^{WAF1/CIP1}$ mediates 1,25-VD triggered G0/G1 cell cycle arrest [9,10]. In addition to antiapoptotic protein and $p21^{WAF1/CIP1}$, the molecules involved in the antiproliferative effect of 1,25-VD also include, but are not limited to, vitamin D receptor (VDR), androgen receptor (AR), and p53. Therefore, the sensitivity to 1,25-VD depends on the composition and activity of these molecules [11–13]. Among PCa cell lines tested, the androgen-responsive cell line, LNCaP, is the most responsive while androgen-independent cell lines, DU 145 and PC-3 show less response to 1,25-VD treatment. Overall, the antiproliferative effect of 1,25-VD involves multiple signals regulating cell cycle and apoptosis, and coordination of these signaling networks determines the sensitivity to 1,25-VD of various PCa cell lines.

The cooperative effect between 1,25-VD and paclitaxel, another member of the taxane family, has been reported [14]. The mechanism is that pre-treatment of 1,25-VD reduced expression of $p21^{WAF1/CIP1}$,which sensitizes the cytotoxic response to paclitaxel in PC-3 cells. In that study, extremely high concentrations of 1,25-VD were used (5 μM), which is difficult to reach in the clinic. Therefore, we first titrated the antiproliferative effect of 1,25-VD and DX on PC-3 and LNCaP cells to select the optimal concentration in order to gain cooperative effect. We then investigated the cooperative effects and mechanistic actions of DX and 1,25-VD in LNCaP and PC-3 cells, which were selected as models representing the androgen-responsive and -independent PCa cells, respectively, for comparison. The sensitivity to cytotoxic chemotherapy agents in cancer cells can be modulated by drug resistance proteins, including P-glycoprotein (Pgp), the MRP family, and ABCG2 [15]. The expression level of MRP in PCa has been shown to correlate with the Gleason score [16,17]. Therefore, we further investigated whether 1,25-VD regulatesd the expression of drug resistance proteins to sensitize cells in response to DX.

2. Materials and methods

2.1. Cell proliferation assay

Cells were seeded in 24-well tissue culture plates in RPMI-1640 containing 10% FBS. After incubation for 24 h, the medium was replaced with fresh medium containing 10% FBS and cells were treated as indicated in figure legends. The final ethanol concentration was 0.1%. Cell proliferation was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer's procedure (Sigma, St Louis, MO). Briefly, the stock solution of MTT (5 mg/ml PBS) was added into each well at a 10-fold dilution. After 2 h incubation at 37 °C, the stop solution was added to extract the formazan product and the absorbance was recorded.

2.2. Flow cytometric analysis

LNCaP and PC-3 cells were seeded in 60-mm dishes at a density of 10^5 cells and 2×10^4 cells, respectively. After treatment, both attached and floating cells were harvested and stained with Annexin V-PE according to the manufacturer's procedure (BD Bioscience, San Diego, CA). The PE positive cell population representing apoptotic cells was determined by using the FACScan flow cytometer.

2.3. Western blot analysis

Total cell lysates were prepared by lysing cells in ice-cold RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS in PBS). The protein concentration was evaluated with the Bio-Rad reagent kit. For analyses, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in TBST (10 mM Tris–Cl/pH 7.4, 150 mM NaCl, 0.05% Tween20) containing 5% nonfat dry milk for 1 h at room temperature. Membranes were probed with primary antibodies against Bax (Santa Cruz), Bcl-2 (DAKO), multidrug resistance-associated protein-1 (MRP-1) (Santa Cruz, Santa Cruz, CA), and β-actin (Santa Cruz), and then the secondary antibodies (Santa Cruz) in TBS. The immunoreactive bands were visualized by chemiluminescence (Amersham, Piscataway, NJ), or by the 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium phosphatase substrate (Bio-Rad Laboratories, Hercules, CA). Protein expressions were quantified using a Versa-Doc gel documentation system (Bio-Rad).

2.4. RT-PCR and quantitative PCR(Q-PCR) assay

Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA). RT-PCR was carried out by reverse transcription with the Super Script II kit (Invitrogen). Q-PCR was performed with SYBR Green PCR Master Mix on an iCycler IQ multi-color Q-PCR detection system (Bio-Rad). Primer sequences were β-actin: sense 5′-TGTGCCCATCTACGAGGGGTATGC-3′ and anti-sense 5′-GGTACATGGTGGTGCCGCCAGACA-3′; MRP-1: sense 5′-GCTGAGTTCCTGCGTACCTATGC-3′ and anti-sense 5′-TGTTGTGGTGCCTGCTGATGTC-3′. The PCR was performed as follows: initial denaturation at 95 °C for 10 min, and 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. ΔCT values were calculated by subtracting the cycle threshold (CT) value from the corresponding β-actin (internal control) CT value from each time point. Then relative amounts were calculated by comparing the ΔCT value of the treated group to the ΔCT value of the control group.

2.5. 5-Carboxyfluorescein (5-CF) accumulation assay

MRP function was measured by modified 5-CF accumulation assay as described previously [18]. Briefly, cultured cells were incubated with DMSO or 200 μM indomethacin for 30 min then exposed to 2 μM 5-CF diacetate for another 30 min. After rinsing three times with ice cold phosphate-buffered saline, cells were lysed using Triton X-100. 5-CF cellular accumulation was then evaluated using a spectrofluorimeter (Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths were 492 and 518 nm, respectively.

3. Results

3.1. Dosage titration of 1,25-VD and DX in PC-3 and LNCaP cells

In order to optimize the dose for 1,25-VD and DX combination treatment, we first carried out dosage titration of each compound to demonstrate their independent effect on PCa cells. PC-3 and LNCaP cells were treated with five different concentrations of 1,25-VD from 1 to 100 nM for 6 days. On day 6, the growth inhibitory effect of 1,25-VD on LNCaP was around 10–20% higher than PC-3 (Fig. 1a). Although 100 nM 1,25-VD yields less than 40% growth inhibitory effect in treated cells, we did not pursue higher dosage considering the potential toxicity in vivo. Five different concentrations of DX ranging from 0.1 to 10 nM were used to treat PC-3 and LNCaP for 6 days. PC-3 cells are more sensitive to DX than LNCaP with an IC50 at 1.5 nM for PC-3 and 8 nM for LNCaP (Fig. 1b).

3.2. 1,25-VD pre-treatment promoted growth inhibitory effect of DX on PC-3

To test the growth inhibitory effect of the 1,25-VD and DX combined treatment on PCa cells, we first performed growth assays 6 days after simultaneously treating cells with both compounds. The co-treatment of 1,25-VD and DX on PCa cells did not yield a better growth inhibitory effect than their independent treatments (data not shown). This is similar to a previous report where no greater antitumor effect was observed under co-treatment of 1,25-VD and paclitaxel, which is another compound of the taxane family [14]. In another treatment design, cells were pre-treated with 1,25-VD for 2 days only and then treated with DX for another 6 days. Two days pre-treatment of 1,25-VD yielded less antiproliferative effect, compared to Fig. 1a, where

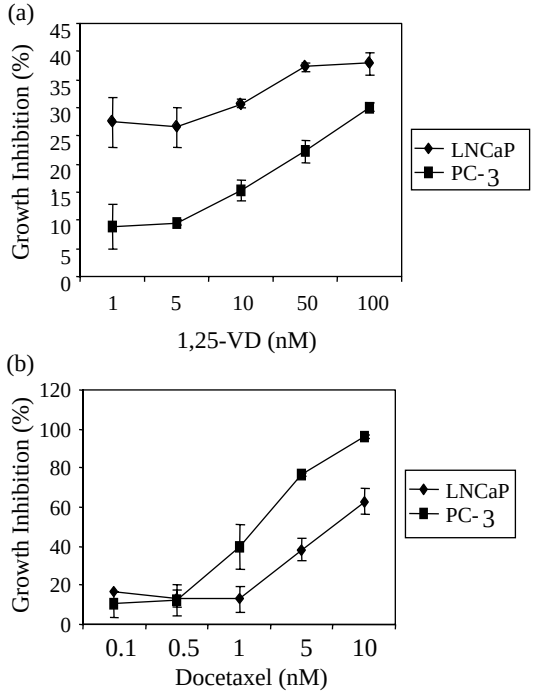

Fig. 1. Dose-dependent growth inhibitory effect of 1,25-VD and DX in PCa cell lines. (a) PC-3 and LNCaP cells were seeded at a density of 2×10^3 cells/well and 10^4 cells/well, respectively, in 24-well plates and cultured in 10% FBS supplemented RPMI. After 24 h, cells were treated with different concentrations of 1,25-VD (panel a), DX (panel b) or their respective vehicles for 6 days. On day 6, the MTT assay was performed to measure the viable cells. The data were expressed as percent of control (in the presence of vehicle only). Each point represents the mean ± standard deviation (SD) of triplicate determinations.

6 days continuous treatment of 1,26VD was performed. Also, the antiproliferative effect of DX was lower compared to the same dosage in Fig. 1b. This is probably because after pre-treatment with EtOH for 2 days, the cell density was higher when the treatment of DX started. Nonetheless, we did see a greater growth inhibitory effect from the combined treatment compared to the single treatment of either compound in PC-3 cells, but not in LNCaP cells (Fig. 2a). Furthermore, we calculated and compared the IC_{50} of DX when treated alone or combined with 1,25-VD pre-treatment in PC-3 cells. DX treatment alone inhibited PC-3 cells growth with an IC_{50} of 4 nM. Antiproliferative effect of DX was promoted when pre-treatment of 1,25-VD was combined with DX where the IC_{50} was reduced to 2.7 nM (Fig. 2b).

3.3. 1,25-VD pre-treatment increased DX induced apoptosis in PC-3

DX inhibits microtubular polymerization, arrests cells in the G2/M phase of the cell cycle and induces apoptosis by Bcl-2 phosphorylation [5]. 1,25-VD is known to arrest cells in G1 phase and also triggers apoptosis by decreasing Bcl-2 expression [7]. Therefore, we examined whether the apoptosis population was increased in combined treatment with 1,25-VD and DX. PC-3 cells were treated with 100 nM 1,25-VD for 2 days followed by 0.5 nM DX treatment for 3 days. High concentrations of 1,25-VD alone increased apoptosis while low concentrations of DX alone had minor effects on apoptosis. Combined treatment of these two compounds yields additive effects in apoptosis induction compared with single treatment (Fig. 3a). In LNCaP, 1 day pre-treatment with 1,25-VD followed by 3 days in normal medium did not cause increased apoptosis compared to EtOH treatment, while DX alone did increase the apoptotic population from 10 to 15% (Fig. 3b). However, combined treatment did not yield a greater apoptotic population compared with DX alone in LNCaP. This result suggests pre-treatment of 1,25-VD did promote the growth inhibition effect of DX in PC-3, but not LNCaP, through increasing apoptosis.

3.4. Bax expression is increased in PC-3 under 1,25-VD and DX combined treatment

Several apoptosis-regulating proteins are known to be involved in 1,25-VD and DX stimulated apoptosis. Here we examined the expression and phosphorylation of Bcl-2, the anti-apoptotic protein, and the expression of Bax, the pro-apoptotic protein. Although the expression and phosphorylation of Bcl-2 did not change in PC-3 under either single or combined treatment, Bax expression was increased under single

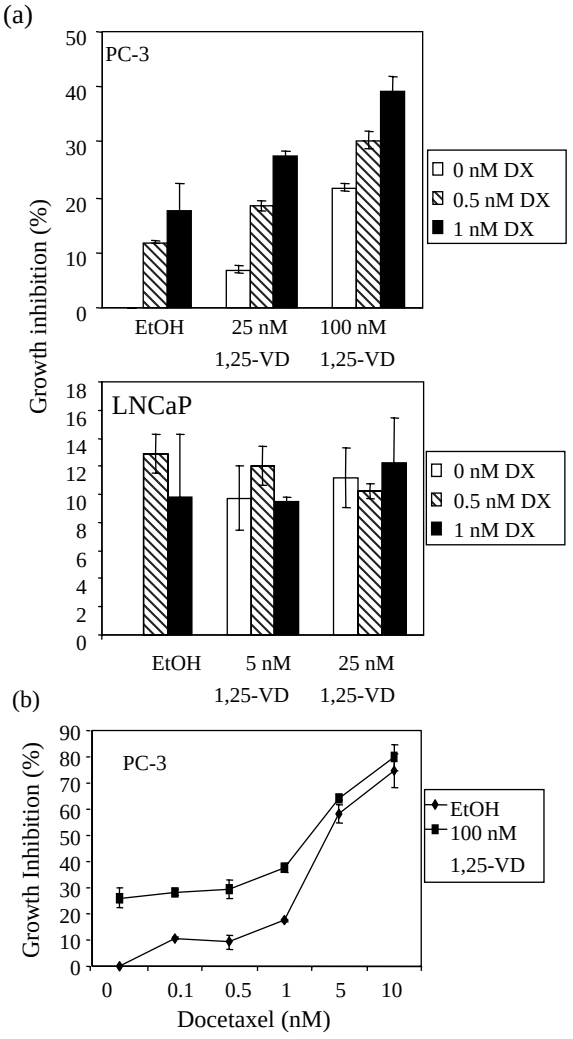

Fig. 2. Pre-treatment with 1,25-VD promotes the growth inhibitory effect of DX in PC-3 but not LNCaP cells. (a) PC-3 and LNCaP cells were seeded at a density of 2×10^3 cells/well and 10^4 cells/well, respectively, in 24-well plates and cultured in 10% FBS supplemented RPMI. After 24 h, cells were treated with EtOH or different concentrations of 1,25-VD for 2 days. Medium was replaced and cells were treated with different concentrations of DX as indicated. After 6 days, the MTT assay was performed to measure the viable cells. Each treatment condition and assay was performed in triplicate, and the percentage of growth inhibition attained by comparing with EtOH treatment was calculated. The mean ± SD was plotted. (b) PC-3 cells were seeded and treated for growth assay as described in (a). The mean ± SD from three independent experiments was plotted against the concentration of DX. IC_{50} of DX with pre-treatments of EtOH or 100 nM 1,25-VD were calculated.

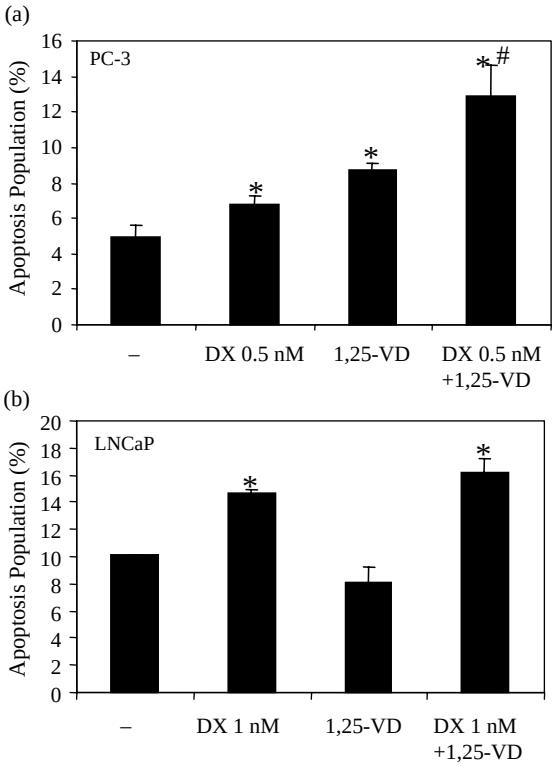

Fig. 3. Pre-treatment with 1,25-VD increases the apoptosis population induced by DX treatment in PC-3 but not LNCaP cells. (a) PC-3 cells were seeded at a density of 2×10^4 in 60-mm dishes and cultured in 10% FBS supplemented RPMI. After 24 h, cells were treated with EtOH or 100 nM 1,25-VD for 2 days. Medium was replaced and cells were treated with or without 0.5 nM DX. After 3 days, the apoptosis populations were assayed and plotted. * $P<0.05$ compared with EtOH treated group by using Student's *t*-test. (b) LNCaP cells were seeded at a density of 10^5 cells in 60-mm dishes and cultured in 10% FBS supplemented RPMI. After 48 h, cells were treated with EtOH or 100 nM 1,25-VD for 1 day. Medium was replaced and cells were treated with or without 1 nM DX. After 3 days, the apoptosis populations were assayed and plotted. * $P<0.05$ compared with EtOH treated group, # $P<0.05$ compared with DX alone treated group by using Student's *t*-test.

treatment of both compound and further increased under combined treatment (Fig. 4, left panel). Since the expression of Bax was further increased while Bcl-2 remained the same in combined treatment compared with single treatment, pro-apoptotic events were further promoted in PC-3 with the combination treatment. On the other hand, the phosphorylation status of Bcl-2 changed dramatically in LNCaP cells with 1,25-VD and DX alone treatment with no further changes in combination treatment (Fig. 4, right panel). No change of Bax expression was observed in LNCaP. Although Bcl-2 phosphorylation increased 2 days after 1,25-VD pre-treatment, apoptosis population did not increase at day three as shown in Fig. 3. This suggests other apoptosis regulating signals are involved. Overall, the correlation between the Bax expression and the additive effect of apoptosis stimulation of 1,25-VD and DX indicates that the pro-apoptotic pathway is involved in the additive effect of combination treatment in PC-3 cells.

3.5. The expression and function of MRP-1 are reduced in 1,25-VD treated PC-3 cells

The cytotoxicity of docetaxel is significantly reduced by the expression of MRP in cancer cells [19]. To test whether 1,25-VD promotes the anti-proliferative effect of DX by modulating the function of MRP, we examined their expression by Q-PCR. The treatment with 1,25-VD alone down-regulated MRP-1 mRNA expression in PC-3 cells (left panel), but not in LNCaP cells (right panel) (Fig. 5a). This reduction also occurred in combined treatment. We then examined the protein level of MRP-1 1 day after changes in mRNA level were observed. The protein expression of MRP-1 in 1,25-VD treated PC-3 cells decreased, and was further decreased after combined treatment (Fig. 5b).

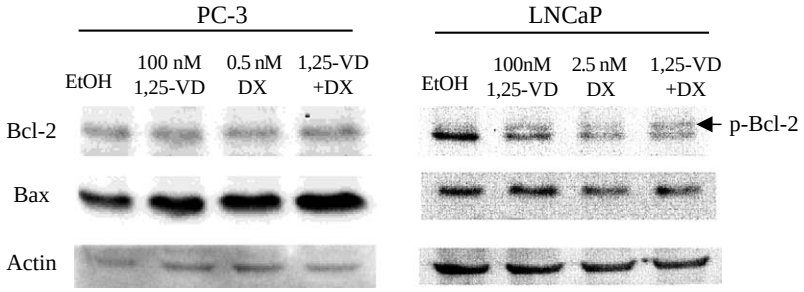

Fig. 4. Pre-treatment with 1,25-VD further increases the protein expression of Bax in DX treated PC-3, but not LNCaP cells. PC-3 and LNCaP cells were seeded at a density of 4×10^4 and 2×10^5 cells, respectively, in 60-mm dishes and cultured in 10% FBS supplemented RPMI. After 24 h, cells were treated with EtOH or 100 nM 1,25-VD for 1 day. Medium was replaced and cells were treated with 0.5 nM or 2.5 nM DX for another 2 days. Cells were then lysed and proteins were harvested for detection of Bcl-2, Bax, and actin by the Western blotting assay.

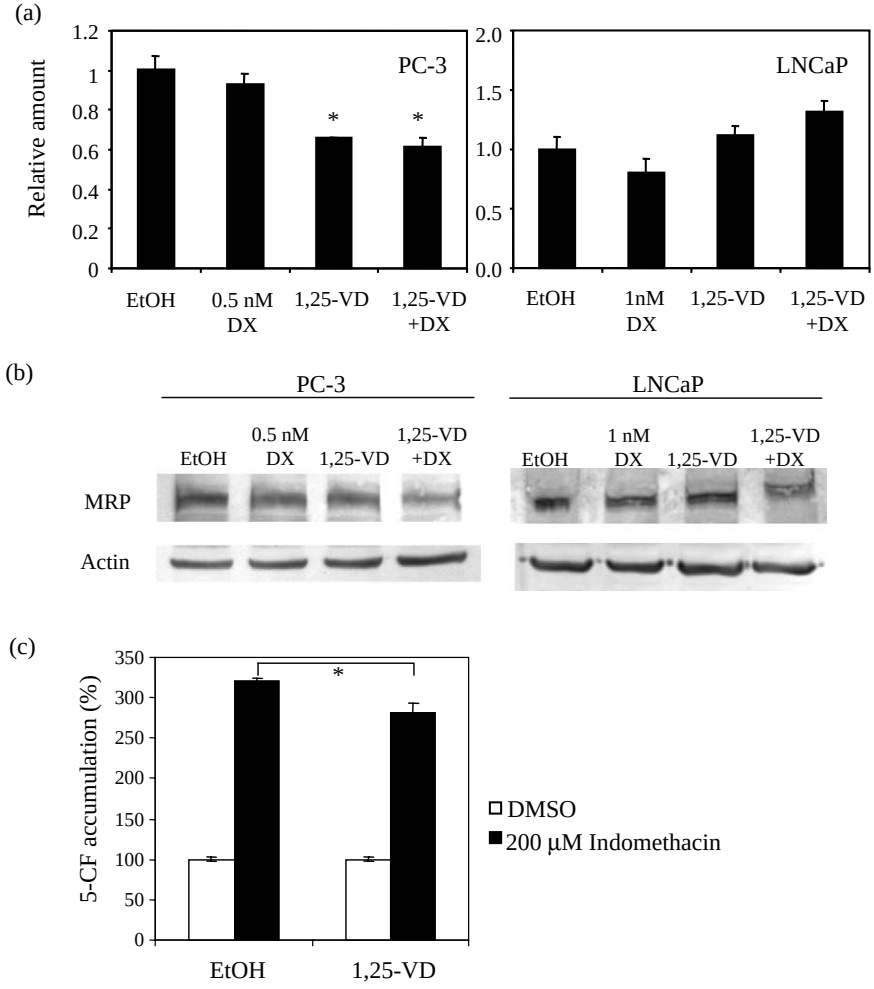

Fig. 5. The expression and function of MRP-1 in PC-3 after DX, or 1,25-VD alone, or combined treatment. (a) PC-3 and LNCaP cells were seeded at a density of 4×10^4 and 2×10^5 cells, respectively, in 60-mm dishes, then cultured in 10% FBS supplemented RPMI. After 24 h, PC-3 cells were treated with EtOH or 100 nM 1,25-VD for 2 days. Forty-eight hours after seeding, LNCaP cells were treated with EtOH or 100 nM 1,25-VD for 1 day. Medium was replaced and cells were treated with or without DX for 1 day. RNA was then harvested for detection of MRP-1 by Q-PCR. Each treatment condition and assay was performed in triplicate. The relative amount was measured by comparing with no treatment after being normalized by actin expression. The mean ± SD was plotted. * $P<0.01$ compared to EtOH treatment by using Student's *t*-test. (b) PC-3 and LNCaP cells were seeded and treated as described in (a), except that cells were treated with or without DX for 2 days before harvesting protein. 100 μg protein from total lysate of PC-3 cells and 60 μg of LNCaP cells were loaded on 6% SDS-PAGE. The expression of MRP-1 and actin were detected by Western blotting. (c) PC-3 cells were seeded at a density of 2×10^4 cells/well of 24-well plate. After 24 h, cells were treated with EtOH or 100 nM 1,25-VD for 48 h and the MRP function assay was performed. The accumulated 5-CF in cells was extracted and measured by fluorescence reader. The 5-CF amount in DMSO treated cells was set as 100%. The relative 5-CF amount was calculated and plotted. * $P<0.01$ compared to EtOH treatment in the presence of indomethacin by using Student's *t*-test.

We next determined whether this decreased expression reflected a reduced function of MRP-1. A previously described 5-CF accumulation assay was used to study the functional activity of MRP [18]. In this assay, the nonfluorescent 5-CF diacetate passively and rapidly diffuses into cells and is converted to the fluorescent anion 5-CF by intracellular esterases. 5-CF is effluxed from cells by the MRP family of transporters. The increased accumulation of 5-CF in the presence of a specific blocker for MRP, indomethacin, represents functional MRP in cells. In Fig. 5c, 1,25-VD treated PC-3 cells had less accumulation of 5-CF (175% increase) in the presence of indomethacin compared to EtOH treated cells (208% increase) indicating the reduced function of MRP. This suggests that by pre-treatment with 1,25-VD, the MRP-1 expression and

function are decreased, which results in accumulation of DX in cells and potentiates the cytotoxicity of DX.

4. Discussion

In advanced PCa patients, the options of treatment are few and prognosis is poor in most of cases. Recently, two phase III studies using DX combined with prednisone or estramustine showed increasing survival in hormone refractory PCa patients [20,21]. In addition, preclinical and clinical phase II studies using combined treatment with 1,25-VD and DX showed promising results in inhibition of PCa cell growth [3,22]. In order to facilitate the cooperative effect of combinated treatment and to benefit future therapeutic design, we investigated the mechanisms by which 1,25-VD pre-treatment can promote the cytotoxicity of DX in PCa cell lines. LNCaP and PC-3 cells were chosen for this investigation to represent the androgen-responsive PCa, and HRPC, respectively. The higher sensitivity to 1,25-VD in LNCaP than in PC-3 is well documented with studies showing the presence of AR or the loss of p53 function are parts of mechanisms that attenuate the effect of 1,25-VD [12,13]. In contrast, the higher sensitivity to DX in PC-3 cells might be due to the rapidly proliferating characteristic of PC-3 cells increasing the opportunity for DX to block microtubule function during mitosis, or due to the lower MRP-1 expression level in PC-3 (data not shown) that results in accumulation of DX in PC-3 cells.

When pre-treated with 1,25-VD, the antiproliferative effect of DX was promoted in PC-3 cells, but not in LNCaP cells. Both 1,25-VD and DX exert antiproliferative effects through interfering with the cell cycle and apoptosis. After dissecting the effect of combined treatments in cell cycle and apoptosis, we did not detect further G0/G1 or G2/M arrest (data not shown). However, we did observe enhanced apoptotic populations in PC-3 cells under combined treatment with 1,25-VD and DX. This was confirmed by the detection of increasing amounts of the pro-apoptotic protein, Bax, in cells treated with either agent, and a further increase in combined treatment. The increase of Bax protein in retinoblastoma cells by 1,25-VD treatment has been reported previously [23], but not in PCa cell lines, LNCaP or ALVA-31 [7]. We also did not observe Bax increases in 1,25-VD treated LNCaP cells, but did so in PC-3 cells. As for the anti-apoptotic protein, Bcl-2, we did observe the phosphorylation in 1,25-VD or DX treated LNCaP cells, but not in PC-3 cells. The reasons behind such discrepancies in regulating apoptosis signals among PCa cells by 1,25-VD and DX requires further investigation.

Although taxanes are the major substrates of Pgp, MRP did promote modest desensitization toward taxanes [24,25]. In our study, no reduction of Pgp expression was observed in PC-3 cells under combination treatment (data not shown). However, we did see a reduced MRP expression in RNA and protein level in PC-3 cells under 1,25-VD and DX alone and in combined treatment. The discrepancy observed in the drug-induced reduction of MRP-1 expression between LNCaP and PC-3 cells might be contributed by the status of functional p53 in the cells. Since the promoter of MRP can be activated by mutant p53 [26], and LNCaP cells express functional, wild type p53, while PC-3 cells express non-functional, truncated p53 [27], it is therefore possible that the suppressive effect of p53 is absent in PC-3 cells so that 1,25-VD is able to regulate MRP-1.

The application of Pgp inhibitor in clinical treatment for cancer has been suggested, however, the correlation of MRP expression in cancer is not well studied. The administration of probenecid, a MRP inhibitor, increased the accumulation of methotrexate and folate analogues in tumor cells, hence enhancing their antitumor efficacy [28,29]. However, probenecid and other MRP inhibitors such as ofloxacin, erythromycin, and rifampicin also increase the expression of MRP [30]. In addition, the physiological functions of the MRP family, including the inflammatory process, hepatobiliary elimination of bilirubin glueuronide, and protecting certain tissues from toxic agents, have to be taken under consideration when applying inhibitors in the clinic [31].

In summary, we confirmed that combination treatment with 1,25-VD enhanced the antiproliferative effect of DX and identified that a potential mechanism for 1,25-VD to sensitize cell response to DX in the PC-3 cell line is by down-regulating MRP-1 expression and function. Other MRP-1 substrates, such as vinca alkaloids, methotrexate, and camptothecins, are potential candidates for combination treatment with 1,25-VD in PCa. However, the mechanism of how 1,25-VD regulates MRP and how this effect varies among different cell lines needs to be investigated in order to maximize the benefit of such combination treatment in patients. Whether 1,25-VD may serve as a better option in chemosensitization compared to MRP inhibitors depends on its selectivity among cancer and normal cells.

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1α, 25-dihydroxyvitamin D_3 suppresses interleukin-8-mediated prostate cancer cell angiogenesis

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Angiogenesis is an essential step in initial tumor development and metastasis. Consequently, compounds that inhibit angiogenesis would be useful in treating cancer. A variety of antitumor effects mediated by 1α, 25-dihydroxyvitamin D_3 (1,25-VD) have been reported, one of which is anti-angiogenesis; however, detailed mechanisms remain unclear. We have demonstrated that 1,25-VD inhibits prostate cancer (PCa) cell-induced human umbilical vein endothelial cell migration and tube formation, two critical steps involved in the angiogenesis. An angiogenesis factor, interleukin-8 (IL-8), secreted from PCa cell was suppressed by 1,25-VD at both mRNA and protein levels. Mechanistic dissection found that 1,25-VD inhibits NF-κB signal, one of the most important IL-8 upstream regulators. The 1,25-VD-mediated NF-κB signal reduction was shown to result from the blocking of nuclear translocation of p65, a subunit of the NF-κB complex, and was followed by attenuation of the NF-κB complex binding to DNA. The role of IL-8 in PCa progression was further examined by PCa tissue microarray analyses. We found that IL-8 expression was elevated during PCa progression, which suggests that IL-8 may play a role in tumor progression mediated through its stimulation on angiogenesis. These findings indicate that 1,25-VD could prevent PCa progression by interrupting IL-8 signaling, which is required in tumor angiogenesis, and thus applying vitamin D in PCa treatment may be beneficial for controlling disease progression.

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer deaths among North American men. Although PCa is initially suppressed efficiently by medical or surgical castration, many patients treated with androgen ablation develop disease progression, and tumors eventually become hormone refractory, for which no therapy has yet demonstrated a definitive survival advantage. Therefore, the need for more options in the treatment of hormone refractory PCa is obvious.

Angiogenesis, the formation of new blood vessels from an existing vascular bed, is a crucial step in the progression of tumor growth, invasion and metastasis, and its inhibition is a putative therapeutic target (1). Angiogenesis occurs physiologically in processes including wound healing, embryogenesis and the ovulatory cycle, and in pathological states including rheumatoid arthritis, diabetic retinopathy and cancer progression (2). Initiation of angiogenesis (angiogenic switch) is controlled by local hypoxia that induces the synthesis of angiogenic factors that can activate signal pathways and transcription for endothelial cell structural reorganization. Endothelial cell reorganization is a multi-step process that includes degradation of vascular basement membrane by matrix metalloproteinases (MMPs), sprouting, elongation, migration and proliferation of endothelial cells followed by the association of endothelial cells into new tubular channels (3). These processes are tightly controlled through a balance of positive and negative regulatory factors.

Epidemiological evidences have suggested that low exposure to sunlight and vitamin D deficiency might be risk factors for PCa (4). 1α, 25-dihydroxyvitamin D_3 (1,25-VD), the active metabolite of vitamin D, has been shown to inhibit vascular endothelial growth factor (VEGF) induced endothelial cell tube formation *in vitro* and reduce vascularization of tumors derived from VEGF-overexpressed MCF-7 breast cancer cells xenografted into mice (5). These results indicate that 1,25-VD inhibits angiogenesis *in vitro* and *in vivo*; however, the detailed mechanisms still largely remain unknown.

Large-scale profiling of the effects of 1,25-VD on gene expression in human head and neck squamous cell carcinoma revealed that 1,25-VD downregulates interleukin-8 (IL-8), an angiogenic factor (6). The human IL-8 cDNA encodes a 99 amino acids protein, and cleavage to a 72 amino acid form is required for full activation of IL-8. IL-8 can form non-covalent dimers in solution, but dimerization is not essential for biological activity (7). IL-8, also known as CXC ligand-8 or monocyte-derived neutrophil chemotactic factor, is a member of the CXC chemokine family, and was initially identified as a regulator for the recruitment and trafficking of leukocytes, particularly neutrophils, to the sites of inflammation (8). IL-8 can induce the adhesion and migration of neutrophils through the endothelium (9), and neutralizing IL-8 inhibits neutrophil infiltration and tissue damage in several types of inflammation, suggesting a causal role of IL-8 in inflammatory reactions (10). The involvement of chronic or recurrent inflammation has been suggested in the development of PCa (11). Subsequent studies indicated that IL-8 is a common chemotactic factor involved in the regulation of pathological angiogenesis, tumor growth and metastasis (12). The receptors for IL-8, CXCR1 and CXCR2, are expressed variety of normal and tumor cells

Abbreviations: 1,25-VD, 1α, 25-dihydroxyvitamin D_3; BPH, benign hyperplasia; CM, conditioned media; ELISA, enzyme-linked immunosorbent assay; HG, high-grade adenocarcinoma; HUVEC, human umbilical vein endothelial cells; LG, low-grade adenocarcinoma; IHC, immunohistochemistry; IL-8, interleukin-8; MK, midkine; MMP, matrix metalloproteinase; N, normal; PCa, prostate cancer; PCR, polymerase chain reaction; PIN, prostatic intraepithelial neoplasia; TGF-β, transforming growth factor-β; TMA, tissue microarray; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

(13,14). Serum levels of IL-8 are elevated in PCa patients, with levels increasing as PCa progresses, suggesting that IL-8 could be a potential independent predictor for PCa (15). Therefore, the suppression of IL-8 production might be able to delay PCa progression.

In this present study, we investigated how 1,25-VD inhibits the PCa cell secreting angiogenic factors, with the focus on IL-8 signals, to suppress angiogenesis. Our study suggested that 1,25-VD exerts anti-angiogenic effects by modulating IL-8 signals to control tumor growth and could potentially be used in the management of advanced PCa.

Materials and methods

Cells, plasmids and materials

1,25-VD was the generous gift of Dr Lise Binderup from Leo Pharmaceutical Products (Ballerup, Denmark), and the IL-8 promoter construct was kindly provided by Dr Antonella Casola from University of Texas Medical Branch. NF-κB reporter construct, p65, and dominant negative inhibitor of NF-κB (mIκB) expression plasmids were kindly provided by Dr Edward Schwarz from University of Rochester. The anti-p65 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); recombinant human IL-8 and anti-IL-8 antibody (for neutralizing) were purchased from R&D systems (Minneapolis, MN). Anti-IL-8 antibody for immunohistochemical (IHC) studies was purchased from Biosource (Camarillo, CA). Tumor necrosis factor-α (TNF-α) was purchased from Calbiochem (San Diego, CA). Human umbilical vein endothelial cell (HUVEC) were obtained from Dr Patricia Simpson-Haidaris from University of Rochester, and HUVEC culture media (Medium 200) was obtained from Cascade Biologics (Portland, OR). LNCaP, PC-3, DU145 and RWPE-1 cells were obtained from the American Type Culture Collection (Manassas, VA), and HPr-1 cells were a generous gift of Dr Yong-Chuan Wong from the University of Hong Kong. Cell culture medium (RPMI-1640 for LNCaP, PC-3 and DU145 cells; keratinocyte serum-free medium for RWPE-1 and HPr-1 cells) was obtained from Gibco BRL (Carlsbad, CA).

Prostate tissue microarray (TMA)

Over a 2-year period (2002–2003), many prostatic adenocarcinoma cases were reviewed at the University of Rochester Medical Center, Strong Memorial Hospital, and 80 cases were selected for microarray. Areas for sampling were designated as normal (N), benign hyperplastic (BPH), prostatic intraepithelial neoplasia (PIN), low-grade adenocarcinoma (LG) and high-grade adenocarcinoma (HG). Tumors were classified as follows: Gleason pattern 1, 2 and 3 were labeled LG, and Gleason pattern 4 and 5 were labeled HG. A total of 50 N, 82 BPH, 35 PIN, 104 LG and 82 HG areas were chosen for sampling, averaging 4–6 cores per sample (16). The TMA was constructed using a manual tissue arrayer (Beecher instruments; Sun Prairie, WI). Core samples were retrieved from the selected region in each donor paraffin block and transferred to a receiver paraffin block. Tissue cores were 0.6 mm in diameter and 1.0–3.0 mm in length, depending on the tissue thickness in the donor block. The array blocks were then incubated 10 min at 37°C to improve adhesion between cores and paraffin of the recipient block. A section from each block was cut, stained and reviewed to ensure quality control. Sections for IHC were placed on charged glass slides. Two array blocks were constructed, one containing N, BPH and PIN cores, the other containing LG and HG adenocarcinoma cores. Each core was examined under a light microscope and separately scored. Cores that had <50% of original tissue present were disregarded. Scoring included percentage of staining and staining intensity (0, 1+, 2+, 3+), and results were recorded as positive (≥5%, 2+ or 3+) or negative (<5% staining or staining intensity <2+). Data obtained were analyzed using Fisher's exact test (17).

Preparation of conditioned media (CM) and measurement of IL-8 by enzyme-linked immunosorbent assay (ELISA)

We seeded 1×10^5 cells/well in 24-well plates and allowed them to attach overnight, and then treated them with either vehicle or indicated concentrations of 1,25-VD for 24 h. The culture medium was then removed, and the cell layers were washed and incubated with serum-free medium. The CM were collected 24 h after incubation and normalized with cell number for subsequent experiments. IL-8 amounts were assayed by ELISA kit according to the manufacturer's suggested procedures (R&D Systems, D8000C).

In vitro tube formation assay

1×10^4 HUVEC were plated on a growth factor-reduced Matrigel (BD Biosciences; Bedford, MA) coated 96-well plate in serum-free medium, medium containing IL-8 or PCa CM. Following 6 h of incubation, the plate was examined for tube formation under a microscope and photographed. For each treatment, three images were captured and the length of tubes formed was quantified using ImageJ (NIH Image; http://rsb.info.nib.gov/ij/).

Cell migration assay

Cell migration assay was performed as described previously (18). Briefly, 5×10^4 HUVEC were seeded to Matrigel-coated inserts (BD Labware; Bedford, MA) in serum-free medium, medium with IL-8 or PCa CM. After 4 h of incubation, the cells remaining on the top of the Matrigel were removed by a cotton swab and the residual cell amounts were measured by MTT assay.

Gelatin substrate gel zymography

HUVEC were incubated with serum-free medium containing either PBS or IL-8 for 24 h, and then CM were collected and analyzed by gelatin zymography as described previously (18). Briefly, CM were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), under non-reducing conditions, in gels copolymerized with 0.1% gelatin. Following electrophoresis, gels were washed 30 min twice in wash buffer [50 mM Tris (pH 7.4) and 2.5% Triton X-100], then rinsed in incubation buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3] and incubated at 37°C for 18 (short development) or 48 (long development) h. Enzyme activities were visualized by staining with Coomassie blue.

DNA pull-down assay

Oligonucleotides corresponding to the NF-κB site of the IL-8 promoter were synthesized according to published sequences (19). Sequences of the oligonucleotides were as follows: sense 5′-biotin-TCGTGGAATTTCCTCTGA-3′ (−84 to −67) and anti-sense 5′-TCAGAGGAAATTCCACGA-3′ (NF-κB binding site is underlined). Nuclear extracts from LNCaP cells were prepared according to the protocol of Andrews *et al*. (20). For DNA pull-down assays, 50 μg nuclear extracts were incubated with probe for 30 min at room temperature. To capture the complexes, streptavidin-agarose was added, incubated for 1 h at 4°C and then washed three times, eluted from the beads by the addition of 2× laemmli buffer and heating to 95°C for 5 min. Proteins were then separated by 10% SDS–PAGE and analyzed by immunoblot for p65.

Statistical and densitometric analyses

The results are the mean ± standard deviation of values obtained from two or three separate experiments. Student's *t*-test was used in IL-8-neutralizing experiments. ANOVA was used to assess the statistical significance of the differences between control, IL-8-treated and 1,25-VD-treated groups. A statistically significant difference was considered to be present at $P < 0.05$. Autoradiograms/bands were scanned, and the mean density of each band was analyzed by the Quantity One program (Bio-Rad; Hercules, CA). Densitometric data presented below bands are the fold changes compared with control sample band densities.

Real-time polymerase chain reaction (PCR) analysis, cell proliferation (MTT) assay, transient transfection, luciferase assay and western blot analysis

The assays above were performed as described previously (21). IL-8 sense primer 5′-CTCCATAAGGCACAAACTTTCAG-3′ and anti-sense primer 5′-GTCCACTCTCAATCACTCTCAG-3′, and primers for MMP-2, MMP-9 and β-actin were described previously (18).

Results

1,25-VD suppresses the IL-8 expression in human prostate epithelial cells

To define the role of IL-8 in PCa progression, we checked the amounts of IL-8 secretion and mRNA expression in several normal and malignant human prostate epithelial cell lines. As shown in Figure 1, the secretion and mRNA expression of IL-8 were higher in PC-3 and DU145 PCa cells, two androgen-independent and more aggressive human PCa cell lines, than in LNCaP, an androgen-dependent PCa cell line, or in immortalized normal human prostate epithelial cell lines, HPr-1 and RWPE-1. This correlation of IL-8 expression with PCa aggressiveness suggested that IL-8 might be involved in PCa progression.

The inhibition effects of 1,25-VD on PCa cells secreting IL-8 were then examined. As shown in Figure 2, 1,25-VD can suppress IL-8 amounts secreted from PCa cells to different

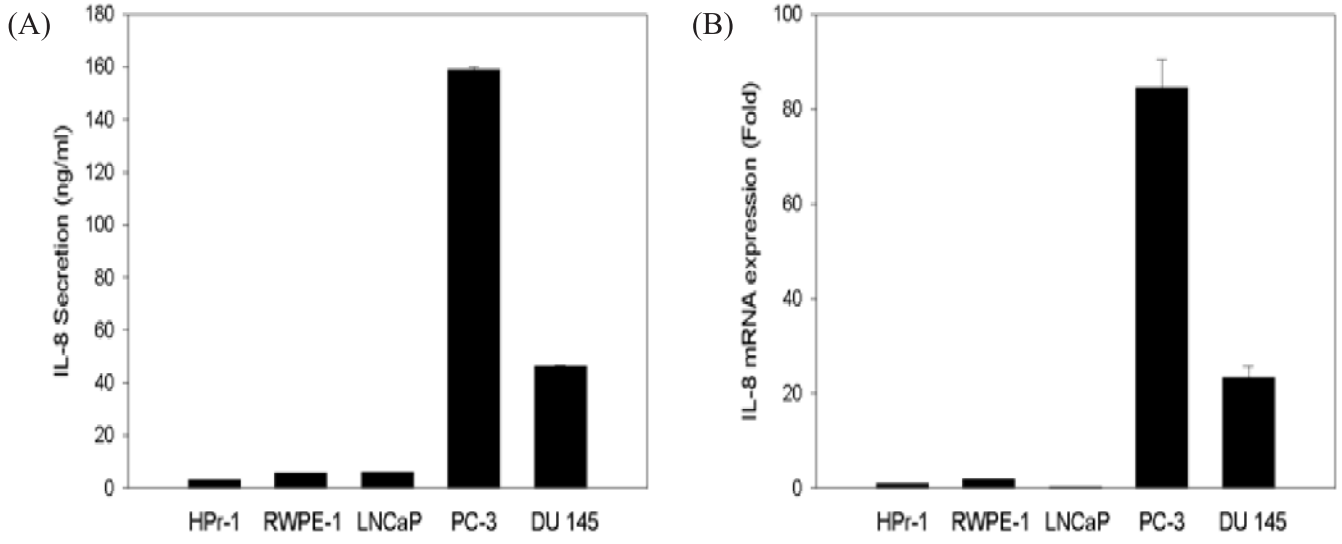


Fig. 1. Examination of the expression levels of IL-8 in human prostate epithelial cell lines. 1×10^5 cells/well were seeded in 24-well plates and were allowed to attach overnight. Cells were incubated with serum-free media for 24 h, or total mRNA were prepared. (**A**) IL-8 secretion in medium was determined by ELISA. (**B**) IL-8 mRNA expression levels were analyzed by real-time PCR. Values represent the fold differences in gene expression relative to HPr-1 cells.

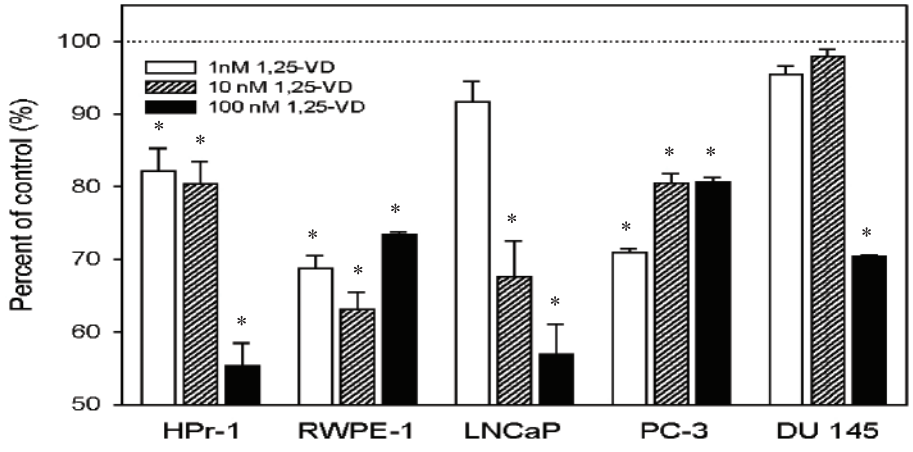


Fig. 2. 1,25-VD suppresses IL-8 secretion in human prostate epithelial cell lines. 1×10^5 cells/well in 24-well plates were seeded and allowed to attach overnight. Cells were treated with either vehicle or indicated concentrations of 1,25-VD for 24 h. The CM were collected to determine IL-8 amount by ELISA. Values represent the percent difference in IL-8 secretion relative to untreated control set as 100. Asterisk indicates significant ($P < 0.05$) differences between control and 1,25-VD-treated groups.

extents, that is, in LNCaP cells, 1,25-VD dose-dependently suppressed IL-8 secretion; in PC-3 cells, a low dose (1 nM) of 1,25-VD was sufficient to suppress IL-8 secretion; however, a much higher dose (100 nM) of 1,25-VD was required to suppress IL-8 in DU145 cells and was possibly due to high endogenous 24-hydroxylase activity (22). Interestingly, we found that 1,25-VD can suppress immortalized normal prostate epithelial cells secreting IL-8, which suggested that 1,25-VD could suppress prostate inflammation, a critical step in tumor initiation.

1,25-VD and IL-8-neutralizing antibody block PCa-stimulated HUVEC tube formation

We applied the HUVEC tube formation, an *in vitro* angiogenesis assay, to examine the effects of IL-8 and 1,25-VD on PCa-stimulated angiogenesis. HUVEC were seeded on Matrigel-coated plates in serum-free medium without and with treatment of IL-8 (1 and 10 ng/ml) for 6 h, and then tube formation was examined. As shown in Figure 3A, IL-8 dose-dependently stimulated HUVEC network formation, as well as the CM from LNCaP, PC-3 and DU145 human PCa cells (Figure 3B), suggesting that PCa cells secrete some angiogenic factors, such as IL-8, to stimulate HUVEC tube formation.

The 1,25-VD effects on the HUVEC tube formation were then examined. We found that 1,25-VD has no direct effect on HUVEC tube formation (data not shown), but it can partially abolish PCa-stimulated HUVEC tube formation. IL-8-neutralizing antibody can inhibit PC-3 and DU145 cells, but not LNCaP cells-induced tube formation. Due to low amounts of IL-8 secretion from LNCaP cells, therefore, 1,25-VD might inhibit the LNCaP-stimulated HUVEC tube formation by

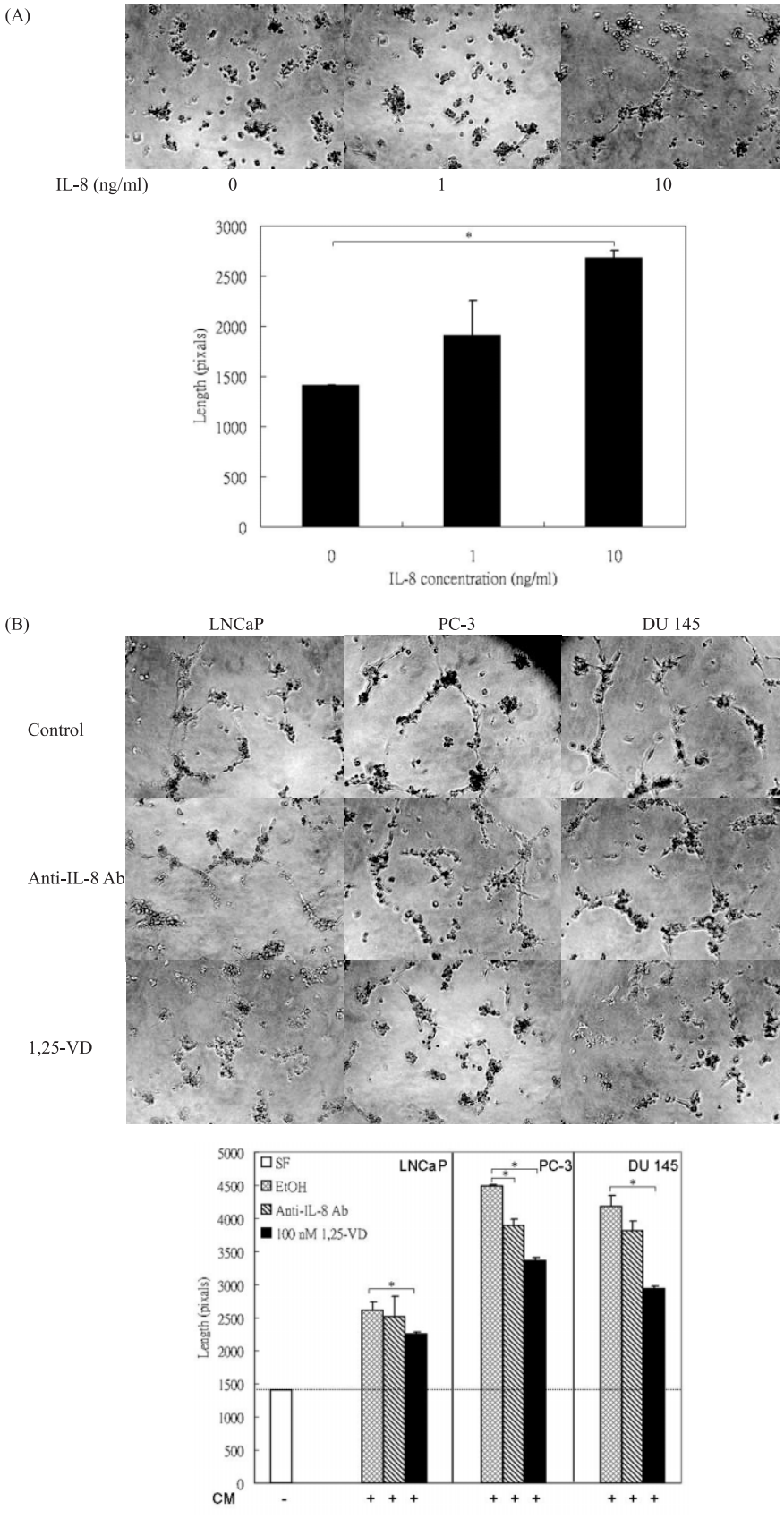


Fig. 3. 1,25-VD and IL-8-neutralizing antibody block PCa CM-stimulated HUVEC tube formation. (**A**) 1×10^4 HUVEC were plated on growth factor-reduced Matrigel-coated 96-well plates with serum-free medium alone, or medium containing indicated concentrations of IL-8. (**B**) 1×10^4 HUVEC were plated on growth factor-reduced Matrigel-coated 96-well plates with serum-free medium. Then, vehicle, 100 nM 1,25-VD-treated or 200 ng/ml IL-8-neutralized PCa CM were added and incubated for another 6 h. The plates were examined for tube formation under microscope and photographed. The length of tubes was quantified using image analysis. Asterisk indicates significant difference ($P < 0.05$).

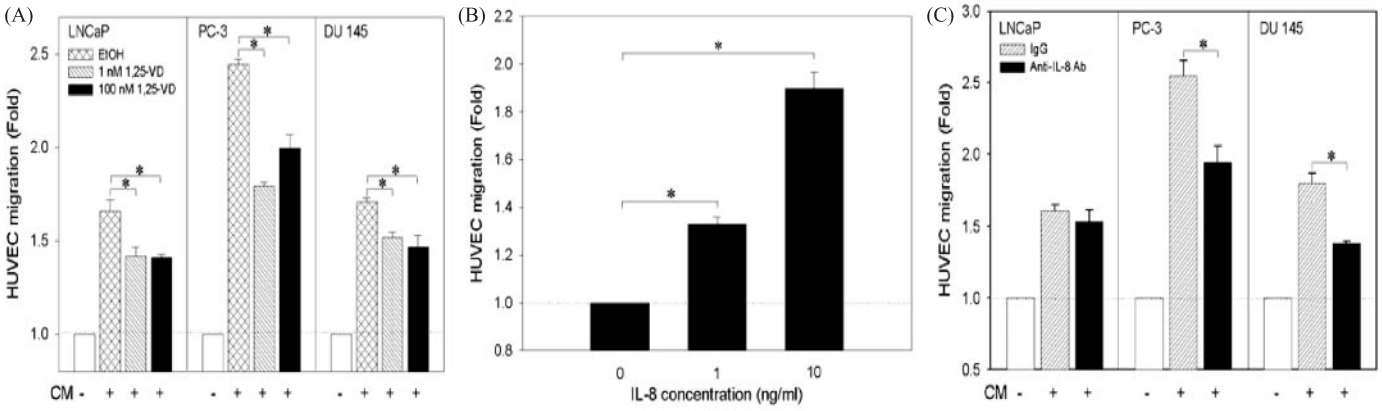


Fig. 4. 1,25-VD and IL-8-neutralizing antibody block PCa CM-stimulated HUVEC migration. 5×10^4 HUVEC were seeded onto Matrigel-coated chamber with serum-free medium. Then, the lower chambers were incubated with (**A**) PCa CM from samples pre-treated with vehicle, or 1,25-VD (1nM or 100 nM), 100 nM), or (**B**) incubated with serum-free medium containing various concentrations of IL-8 or (**C**) incubated with PCa CM pre-treated with IgG, or anti-IL-8 antibody. After 4 h of incubation at 37°C, the cells remaining on the top of the Matrigel were removed by cotton swabs and the residual cells were determined by MTT assays. Values represent the fold increase of migrated cells relative to serum-free medium treated control. Asterisk indicates significant difference ($P < 0.05$).

regulating other angiogenic factors. Together, our data found that IL-8 and other angiogenic factors secreted from PCa cells could stimulate endothelial cell tube formation, and suppression of IL-8 secretion by 1,25-VD and its neutralizing antibody could suppress the HUVEC tube formation and possibly delay PCa progression.

1,25-VD inhibits PCa-stimulated HUVEC migration

Endothelial cell proliferation and migration, two important steps in the process of angiogenesis, were examined. HUVEC were treated with serum-free medium, vehicle- or 1,25-VD-treated PCa cell CM for 4 days, and HUVEC proliferation was determined by MTT assay. The CM from three PCa cell lines can stimulate HUVEC proliferation, compared with serum-free medium. However, 1,25-VD-treated PCa cell CM had no significant effect on HUVEC proliferation compared with vehicle-treated CM (data not shown).

The stimulatory effects of PCa CM on HUVEC migration were then measured. The CM from three PCa cell lines stimulated HUVEC migration from 1.6- to 2.5-fold (Figure 4A, crossed versus open bar), and 1,25-VD-treated CM showed less stimulatory effects on HUVEC migration (Figure 4A, crossed versus striped and black bars). IL-8 enhanced HUVEC migration in a dose-dependent manner (Figure 4B), and IL-8-neutralizing antibody, not IgG control, can partially reverse the CM-stimulated HUVEC migration (Figure 4C).

1,25-VD inhibits PCa-stimulated MMP-9 expression in HUVEC

The effects of 1,25-VD on the expression of MMPs, downstream targets of IL-8 (23), were examined. Real-time PCR analysis demonstrated that treatment of IL-8 in HUVEC induced MMP-9, but not MMP-2 mRNA expression (Figure 5A). Gelatin zymography assay further confirmed that IL-8 stimulated MMP-9, but only slightly enhanced MMP-2 activity in HUVEC (Figure 5B). PCa CM induced the mRNA level of MMP-9, not MMP-2, and 1,25-VD-treated PCa CM showed less MMP-9 induction (Figure 5C). Similar to 1,25-VD treatment, IL-8 antibody reduced the PC-3 and DU145 cells CM-stimulated MMP-9, but not MMP-2, mRNA expression (Figure 5D). Taken together, these data demonstrated that angiogenic factors, including IL-8, secreted from PCa cells can induce MMP-9 expression, which might be able to stimulate HUVEC migration, and this IL-8-mediated MMP-9 induction can be inhibited by 1,25-VD and IL-8-neutralizing antibody.

1,25-VD suppresses TNF-α-induced IL-8 expression in human PCa cell lines

In order to study the underlying mechanism by which 1,25-VD regulates IL-8 expression in PCa cells, TNF-α was used to stimulate IL-8 signals. LNCaP, PC-3 and DU145 cells were treated with or without 1,25-VD for 1 h and then stimulated with TNF-α for another 24 h. We found that TNF-α significantly induced IL-8 secretion (Figure 6A) and mRNA expression (Figure 6B), and that both can be suppressed by 1,25-VD treatment, except in PC-3, where a much lower TNF-α-induced IL-8 secretion and no significant 1,25-VD inhibitory effects were seen. To examine if 1,25-VD regulates IL-8 expression at the transcriptional level, IL-8 promoter reporter (IL-8-Luc) assays were performed. We found that the basal level of IL-8-Luc activity is higher in PC-3 cells than in DU145 and LNCaP cells (Figure 6C), and 1,25-VD can partially inhibit TNF-α-induced IL-8-Luc activity in LNCaP cells, while there is no significant TNF-α induction nor 1,25-VD inhibition effect in PC-3 and DU145 cells (Figure 6D).

1,25-VD inhibits TNF-α-induced p65 translocation in LNCaP cells

So far, no vitamin D response element has been identified in the IL-8 promoter, so we suspect that 1,25-VD might regulate IL-8 expression through cross-talk with other regulatory factors. The NF-κB pathway, one of the most important pathways regulating IL-8 expression, was examined by NF-κB DNA-binding luciferase (NF-κB-Luc) reporter assays. As shown in Figure 7A, the basal level of NF-κB-Luc is higher in PC-3 and DU145 than in LNCaP cells, which corresponds with the IL-8-Luc promoter activity. Next, we applied p65 and mIκB to modulate the NF-κB activities to examine if alteration of NF-κB could affect IL-8-Luc activity. As shown in Figure 7B, p65 stimulates NF-κB-Luc, as well as IL-8-Luc activities, and mIκB suppresses both NF-κB-Luc and IL-8-Luc activities. The induction of NF-κB by p65 was lower in

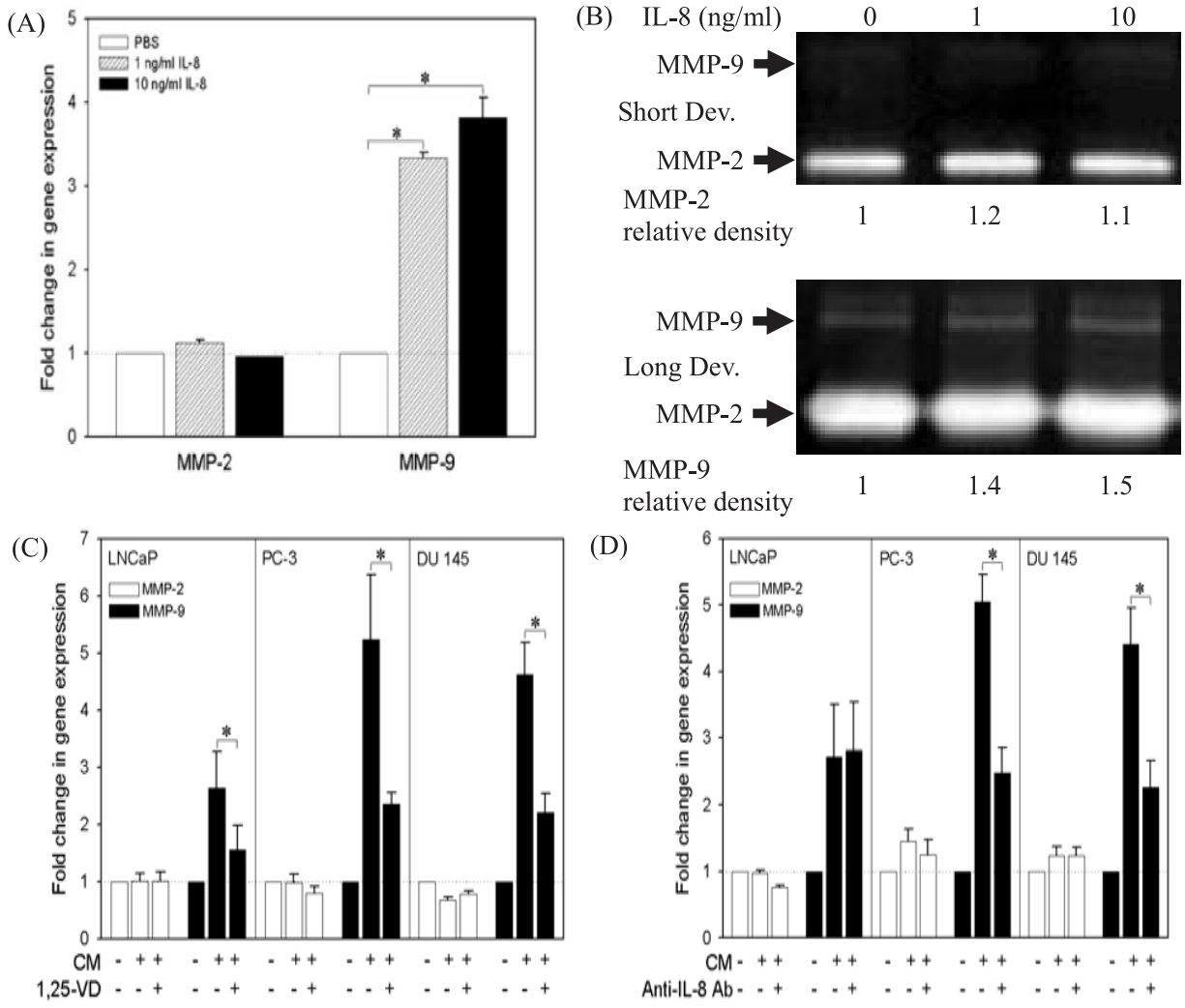


Fig. 5. 1,25-VD and IL-8-neutralizing antibody suppress PCa CM-stimulated MMP-9 expression in HUVEC. Effects of IL-8 on MMP-2 and MMP-9 expressions (**A**) and activities (**B**) in HUVEC. HUVEC were cultured and treated with serum-free medium alone, or medium containing various concentrations of IL-8 for 24 h. Total mRNA and CM were harvested. The MMP-2 and MMP-9 mRNA expression levels (A) were analyzed by real-time PCR, and their activities (B) were determined by zymography [short development time (18 h): upper panel; long development time (48 h): lower panel]. Induction folds were calculated by the expression relative to controls. Effects of 1,25-VD-treated (**C**) and IL-8-neutralized (**D**) PCa CM on MMP-9 expression in HUVEC. HUVEC were cultured and treated with serum-free medium, (C) CM pre-treated with vehicle, or 100 nM 1,25-VD, or (D) CM neutralized by IgG, or anti-IL-8 antibody. Total mRNA was prepared and analyzed by real-time PCR. Values represent the fold increases in gene expression relative to serum-free medium treated control. Asterisk indicates significant difference ($P < 0.05$).

PC-3 and DU145 cells, which might be due to constitutively active NF-κB in these two cell lines so that exogenous transfection of p65 would not induce NF-κB activity further (Figure 7A).

We then examined how 1,25-VD affects NF-κB activity. As shown in Figure 7C, a 56-fold NF-κB-Luc activation by TNF-α treatment was detected in LNCaP cells, and 1,25-VD partially suppressed the TNFα-induced NF-κB-Luc activity. Due to the high basal level of active NF-κB, there is no obvious TNF-α induction of NF-κB activity in PC-3 and DU145 cells, so we mainly focused on the regulation of IL-8 by 1,25-VD in LNCaP cells in the following experiments.

To determine whether 1,25-VD can prevent the NF-κB complex from binding to its corresponding DNA sequence, DNA pull-down assays were performed. Biotin-labeled oligonucleotides, corresponding to the NF-κB binding site in the IL-8 promoter, were used to pull down the NF-κB complex from TNF-α-stimulated LNCaP nuclear extracts, with or without treatment with 1,25-VD. As shown in Figure 7D, TNF-α treatment enhanced the amounts of p65/NF-κB DNA-binding complex, and 1,25-VD decreased the TNF-α-stimulated p65/NF-κB DNA-binding complex. Moreover, p65 nuclear translocation, an essential step for NF-κB activation, induced by TNF-α (Figure 7E, lane 2 versus 1), was retarded by 1,25-VD treatment (Figure 7E, lanes 3 and 4 versus 2). Therefore, we concluded that 1,25-VD could reduce the nuclear translocation of p65 and prevent DNA binding, which consequently suppressed NF-κB-mediated IL-8 transcriptional activity.

Correlation of IL-8 expression with PCa progression

To further investigate the role of IL-8 in PCa progression *in vivo*, we examined IL-8 expression in a large number of prostate carcinoma cases using a TMA. Five different types of prostatic tissue including N, BPH, PIN, LG and HG were collected. As demonstrated in Figure 8, a cytoplasmic staining

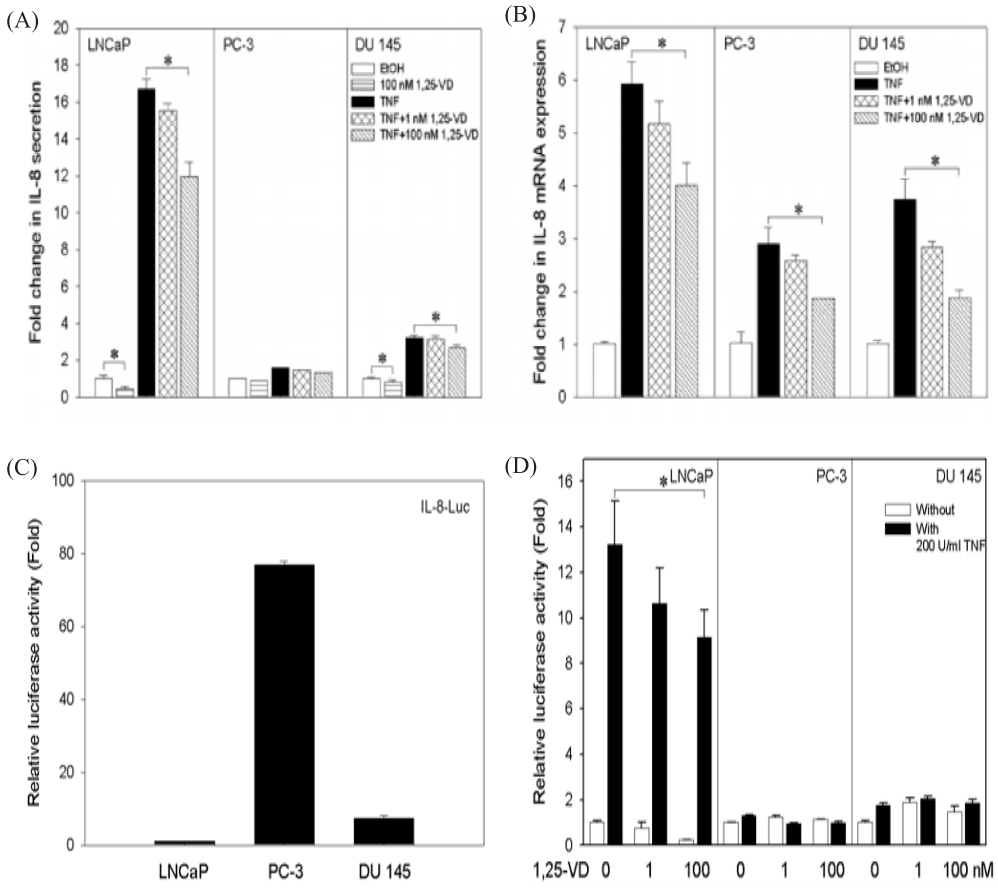


Fig. 6. 1,25-VD suppresses TNF-α-induced IL-8 expression. (**A**) Effects of 1,25-VD on TNF-α-induced IL-8 secretion. Cells were pre-treated with either vehicle or various concentrations of 1,25-VD for 1 h, and then treated with TNF-α (200 U/ml) for 24 h. IL-8 secretion amounts were determined by ELISA. The relative IL-8 secretion was compared with untreated control. (**B**) Effects of 1,25-VD on TNF-α-induced IL-8 mRNA expression. Cells were pre-treated with either vehicle or various concentrations of 1,25-VD for 1 h, and then treated with TNF-α (200 U/ml) for 24 h. IL-8 mRNA levels were determined by real-time PCR. (**C**) The basal IL-8 promoter activity in PCa cell lines. (**D**) The 1,25-VD effects on TNF-α-induced IL-8 promoter activity. PCa cells were transiently transfected with 0.8 μg of IL-8 reporter constructs, and treated with vehicle or various concentrations of 1,25-VD for 1 h, and then treated with TNF-α (200 U/ml) for 24 h. Reporter activities were measured by the Luc assay. The fold induction of Luc activity is presented relative to vehicle treatment. Asterisk indicates significant difference ($P < 0.05$).

was observed in all positive cores, similar to the positive control using endometrial tissue (data not shown). Negative IL-8 staining was shown in one N and one BPH sample (Figure 8A and B) and positive IL-8 cytosolic staining in LG (Figure 8C) and HG (Figure 8D) samples. After reviewing and scoring, we summarized the results as shown in Figure 8E. The positive IL-8 staining was 40% (46 out of 114) in benign tissue cores (normal and BPH), 75% (18 out of 24) in PIN cores and 72% (81 out of 112) in carcinoma cores (LG and HG). Significant increases of IL-8 expressions were found between benign and carcinoma tissues ($P < 0.01$), as well as between benign and PIN tissues ($P < 0.01$). Additionally, there was a significant IL-8 staining increase between LG and HG carcinomas ($P < 0.05$). These results demonstrated that IL-8 expressions correlated with PCa aggressiveness, indicating that IL-8 might serve as a prognostic factor for human PCa.

Discussion

There are several steps in tumor progression that could be regulated by 1,25-VD. First, 1,25-VD is a potent growth inhibitor for cells of epithelial origin or distal metastasis, and this inhibition can be achieved by inducing cell cycle arrest, differentiation or apoptosis (21,24). Second, it reduces tumor metastasis, which involves modulation of proteases (18,25). Third, 1,25-VD has been shown to inhibit angiogenesis of cancer cells (5). In this study, we focused on how 1,25-VD suppresses PCa-stimulated angiogenesis.

Our results have provided several pieces of evidence that suggest that IL-8, one of the most important angiogenic factors secreted by PCa cells, stimulates angiogenesis, and that 1,25-VD could delay PCa progression by suppressing IL-8. First, a positive correlation of IL-8 expression with aggressiveness of PCa was demonstrated in the cell lines and human prostate tissues (Figures 1 and 8). Second, CM from PC-3 and DU145 cells induced more HUVEC tube formation and migration than LNCaP cells (Figures 3B and 4C), which also correlated with IL-8 expression and the aggressiveness of PCa cells. Third, 1,25-VD and IL-8-neutralizing antibody treatment had better inhibitory effects on HUVEC tube formation and migration in PC-3 and DU145 than in LNCaP cells (Figures 3B and 4C), suggesting that 1,25-VD could suppress PCa-induced angiogenesis via inhibition of IL-8, especially in PCa cells with high IL-8 expression.

We noted that neutralizing IL-8 only partially inhibits PCa-stimulated endothelial cell tube formation, migration and MMP-9 expression, compared with 1,25-VD treatment (Figures 3B, 4C and 5D), suggesting that in addition to

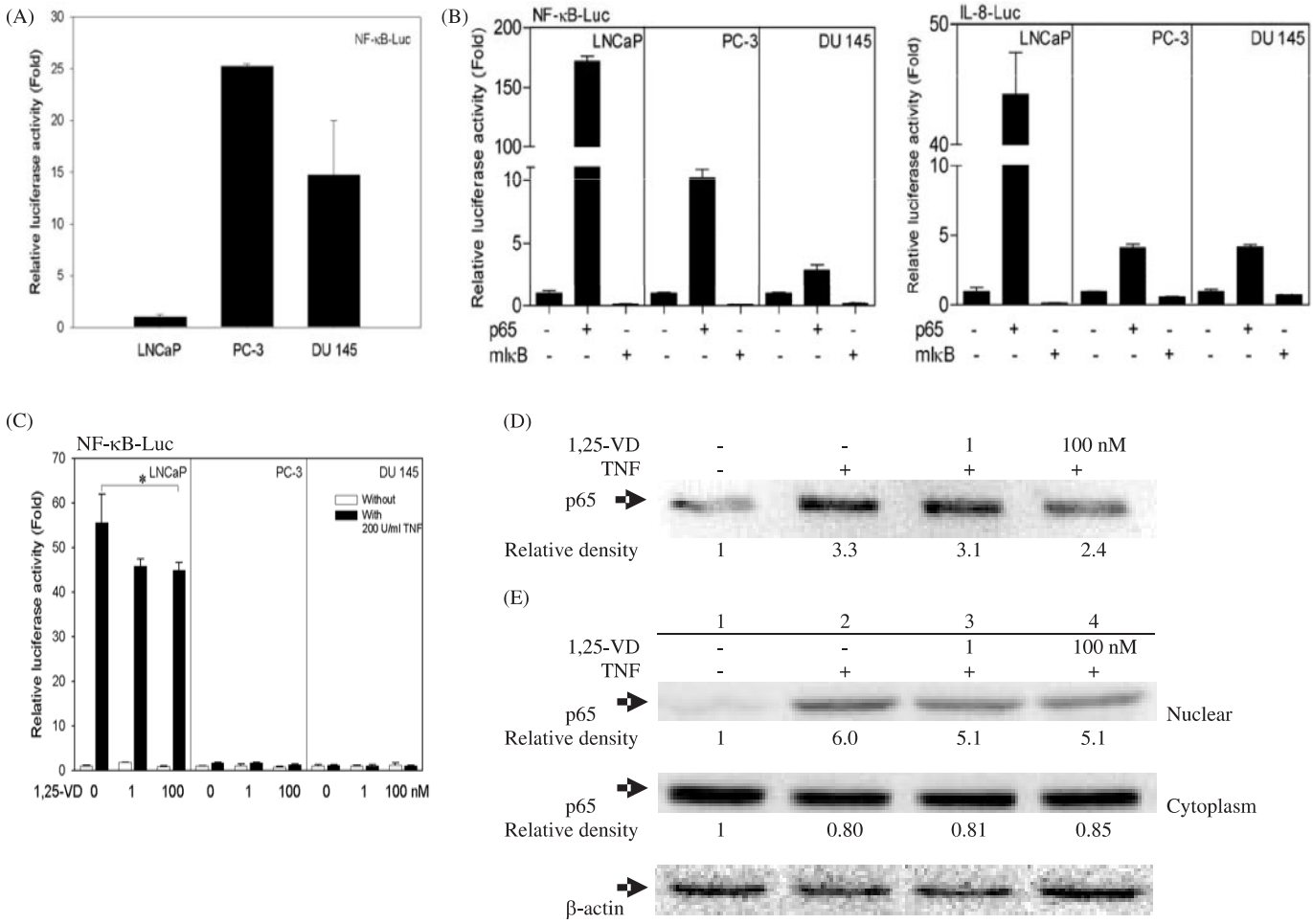
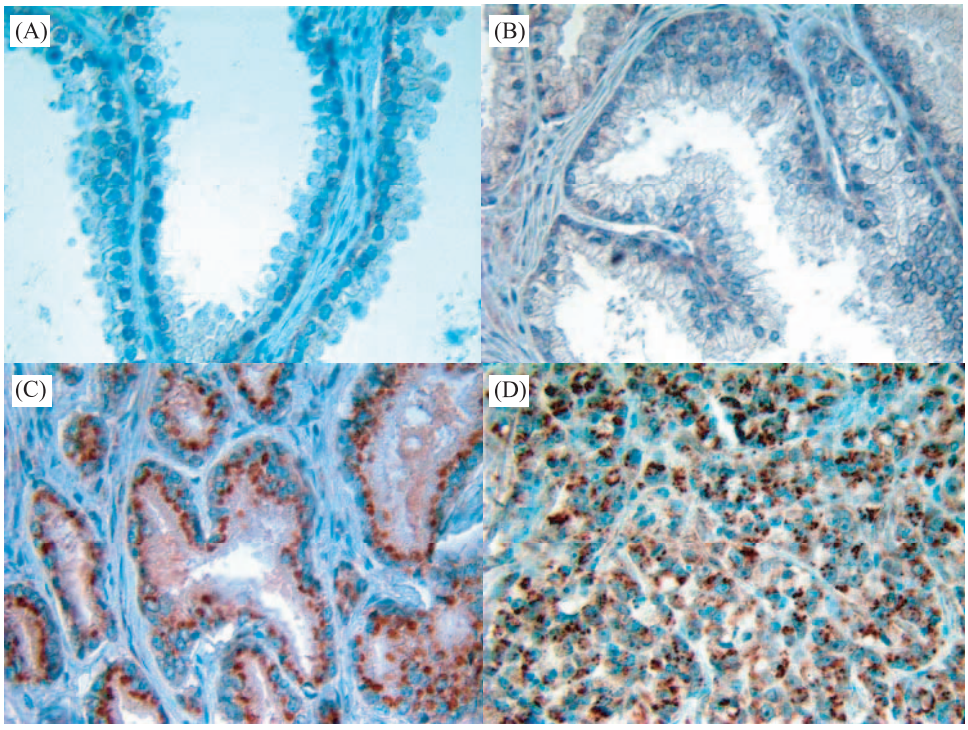


Fig. 7. Regulation of NF-κB activity by 1,25-VD in human PCa cell lines. (**A**) Endogenous NF-κB activity in human PCa cell lines. Cells were transiently transfected with 0.8 μg NF-κB reporter constructs for 24 h. Reporter activities were determined by Luc assay. The fold changes in the NF-κB activity are presented relative to LNCaP cells set at 1. (**B**) Effects of p65 and mIkB on IL-8 promoter activity. Cells were transiently transfected with 0.4 μg NF-κB or IL-8 reporter constructs, and 0.6 μg control vector, p65 or mIkB expression plasmids as indicated for 24 h. Reporter activities were determined by Luc assay. The fold changes in the reporter activity are presented relative to control vector-transfected cells set as 1. (**C**) 1,25-VD moderately suppressed TNF-α-induced NF-κB activity. Cells were transiently transfected with 0.8 μg NF-κB reporter constructs, and treated with vehicle or various concentrations of 1,25-VD for 1 h and then treated with TNF-α (200 U/ml) for 24 h. Reporter activities were determined by Luc assay. The fold changes in the NF-κB activity are presented relative to untreated control set as 1. Asterisk indicates significant difference ($P < 0.05$). (**D**) 1,25-VD suppresses NF-κB DNA binding capacity. Nuclear extracts were prepared from LNCaP cells treated with vehicle or various concentrations of 1,25-VD for 1 h and then treated with TNF-α (200 U/ml) for another 1 h. Fifty micrograms of nuclear extracts were incubated with NF-κB binding DNA probes as described in Materials and methods. After DNA pull-down assay, proteins in the resulting DNA-protein complexes were separated by 10% SDS–PAGE and analyzed by immunoblot for the p65. (**E**) TNF-α-induced p65 translocation is suppressed by 1,25-VD. Cells were treated with vehicle or various concentrations of 1,25-VD for 1 h and then treated with TNF-α (200 U/ml) for another 1 h. Cell lysates were fractionated into nuclear and cytoplasmic fractions, and then analyzed by western blotting with p65 antibody.

IL-8, some other angiogenic factors could be regulated by 1,25-VD. Gene microarray analysis allows global, unbiased evaluation of a broad number of genes and can make the process of studying gene regulation more efficient. By using this technique, large-scale profiling of 1,25-VD effects on gene expression has been conducted in human head and neck squamous cell carcinoma (6), revealing that 1,25-VD upregulates some anti-angiogenic factors, such as bone morphogenetic protein 2A (BMP-2A) and transforming growth factor-β (TGF-β), as well as downregulates some angiogenic factors, such as endothelin-1 (ET-1), chorionic gonadotropin beta subunit, retinoic acid inducible factor midkine (MK), VEGF-related protein, Cyr61 and IL-8. The effects of 1,25-VD on these genes' regulation were also tested by RT–PCR in human PCa cell lines (data not shown). TGF-β regulates cell proliferation, migration, extracellular matrix production and differentiation in a wide variety of cell types (26). TGF-β also inhibits endothelial cell proliferation and induces extracellular matrix deposition, thus controlling the resolution phase of angiogenesis (27). However, stimulatory effects of TGF-β on angiogenesis have also been reported *in vivo* (28). Therefore, the cellular responses mediated by TGF-β in endothelial cell function are complex, being either stimulatory or inhibitory, depending on the differentiation status of cells and cues from the surrounding environment (29). BMPs, other members of the TGF-β superfamily, have been reported to suppress vascular smooth muscle cell proliferation and increase the expression of smooth muscle differentiation markers (30). ET-1 is a potent mitogen for both endothelial cells and vascular smooth muscle cells, and it may indirectly enhance endothelial cell proliferation through stimulation of VEGF production by other cell types (31–33). Elevated ET-1 plasma



(E)

	Positive	Negative	Total	%positive
Normal	13	31	44	**29.55%**
BPH	33	37	70	**47.14%**
Benign	46	68	114	**40.35%**
PIN	18	6	24	**75.00%**
LG	43	24	67	**64.18%**
HG	38	7	45	**84.44%**
Carcinoma	81	31	112	**72.32%**

	p value
Benign vs Carcinoma	0.001
Benign vs PIN	0.002
LG vs HG	0.011

Fig. 8. Elevation of IL-8 expression during PCa progression in TMA analyses. (**A**) A typical example of negative staining of IL-8 in normal prostate tissue sample. (**B**) A typical example of negative staining of IL-8 in BPH tissue sample. (**C**) An intense cytoplasmic staining of IL-8 IHC in LG carcinoma sample. (**D**) An intense cytoplasmic staining of IL-8 IHC in HG carcinoma sample. (**E**) Correlation of IL-8 expression with PCa progression. Data obtained were analyzed using Fisher's exact test. ×400 magnification.

levels have been detected in patients with various tumors including PCa, and levels are increased in metastastic and hormone refractory stages (16). The expression of MK was initially considered to be restricted in embryonic development and the adult brain (34). However, recently, MK has also been shown to be expressed in a range of primary human tumors, and the expression of MK in invasive bladder carcinomas correlates with poor patient survival (35). Cyr61 is a secreted, extracellular matrix-associated, angiogenic regulator, and it can stimulate endothelial cell proliferation and migration *in vitro* and induces angiogenesis *in vivo* (36,37). Cyr61-null mice suffer embryonic death due to loss of vascular integrity in the embryo (38). Furthermore, overexpression of Cyr61 promotes tumor growth in vascularization and is associated with human breast cancer (39). Therefore, the anti-angiogenic effects of 1,25-VD might be not only via the suppression of IL-8 but also via the regulation of other angiogenic factors, which might need further investigations.

The role of chronic or recurrent inflammation in the development of PCa has been suggested (11). IL-8 is a potent chemotactic factor for neutrophils and is well associated with the initiation of an inflammatory response. Ferrer *et al.* (40) found that PCa specimens stained positively for IL-8, whereas BPH and normal tissue exhibited little staining. Serum levels of IL-8 were also elevated by >2-fold in patients with stages A–C PCa in comparison with healthy individuals, and patients with stage D PCa had 4-fold elevation of their IL-8 serum level (15). As we have shown in Figure 8, TMA results also strongly supported that IL-8 expressions were elevated during the PCa progression and that the suppression of IL-8 production might be beneficial for the control of various types of inflammatory reactions or even cancer development.

IL-8 can be regulated both at the transcriptional and post-transcriptional levels. In the 3′-flanking region, *IL-8* gene contains the repetitive ATTTA motif, which is responsible for destabilization of various cytokine mRNAs (41). Also, within the *IL-8* 5′-promoter region, there are potential binding sites for many transcription factors, such as AP-1, AP-2, AP-3, HSE, HNF-1, IRF-1, glucocorticoid receptor (GR), NF-κB, NF-IL-6 and octamer factor that can regulate *IL-8* expression (42). The sequence located at −94 to −70 of the IL-8 promoter is essential for responding to various stimuli, mainly through NF-κB (43). For example, the inhibition of inflammatory cytokine expression by glucocorticoid is GR-dependent via either direct interaction with the p65 subunit of NF-κB or upregulation of NF-κB inhibitor IκBα (44,45). 1,25-VD has been shown to reduce levels of p50 and its precursor p105 and then decrease PMA-induced NF-κB binding to the

IL-6promoter in human lymphocytes (46). 1,25-VD can also partially inhibit NF-κB activity in MRC-5 normal human fibroblasts by targeting DNA binding of NF-κB (47). In our studies, we demonstrated that 1,25-VD downregulates TNF-α-induced IL-8 promoter activity via reduction of p65 nuclear translocation in LNCaP human PCa cell line (Figure 7E). 1,25-VD can significantly reduce IL-8 secretion, but only moderately suppress IL-8 promoter and NF-κB activities, indicating that 1,25-VD might downregulate IL-8 by mechanisms other than the inhibition of NF-κB DNA binding. Therefore, it might be possible that 1,25-VD can also affect the stability of IL-8 mRNA through the ATTTA motif in the 3′-flanking region or other post-transcriptional regulations.

The principal clinical problem of cancer is metastasis. Angiogenesis is a critical step in tumor progression, and its inhibition has obviously become a therapy target. Our study demonstrates that 1,25-VD significantly inhibits human PCa cell-induced endothelial cell migration and tube formation. This inhibition was associated with the suppression of IL-8 secretion from cancer cells. The inhibition of the angiogenic activity of cancer cells by 1,25-VD supports the clinical use of 1,25-VD, either alone or in combination with other chemotherapeutic drugs in the management of advanced PCa.

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